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Award Number: DAMD17-02-1-0584

TITLE: Regulation of BRCA1 Function by DNA Damage-Induced Site-Specific Phosphorylation

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REPORT DATE: June 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2007	Annual Summary	15 May 2002– 15 May 2007

4. TITLE AND SUBTITLE

Regulation of BRCA1 Function by DNA Damage-Induced Site-Specific Phosphorylation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0584

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The University of Texas Health Science Center
at San Antonio
San Antonio, TX 78229-3900

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

BRCA1, a hereditary breast and ovarian specific tumor suppressor, ensures genomic integrity through its control of transcription and repair of damaged DNA. Considerable evidence implicates DNA damage-induced site-specific phosphorylation in the modulation of its biological activity. However, it is not presently clear whether and how the transcription and DNA repair activities of BRCA1 are modulated in response to DNA damage signals. We have engineered and refined a unique combination of biochemical and genetic tools to address this issue. First, we have developed a biochemical means by which to resolve BRCA1-containing complexes involved in transcription from those involved in DNA double-strand break repair. This should render it feasible to identify DNA damage-induced site-specific phosphorylation events with potential functional relevance to the role of BRCA1 in these two processes. Second, we have established fibroblast cultures from brca1-deficient mouse embryos and developed BRCA1-dependent transcription and repair assays based on the use of these cells. This system will expedite the facile and efficient analysis of the effects of targeted BRCA1 mutations at identified or predicted sites of phosphorylation on its transcription and DNA repair activities. Collectively, these studies should illuminate the molecular basis for the caretaker properties of BRCA1.

15. SUBJECT TERMS
Tumor Suppressor, BRCA1, DNA Repair, Transcription, Breast Cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	87	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

Introduction……..1

Body……...1

Key Research Accomplishments……...4

Reportable Outcomes……5

Conclusions……..6

References……..7

Appendices……8

INTRODUCTION

BRCA1, a hereditary breast- and ovarian-specific tumor suppressor, functions in the global maintenance of genome stability, and has been implicated in both transcription and DNA double-strand break repair processes (1,2). Considerable evidence implicates DNA-damage-induced site-specific phosphorylation of BRCA1 as a critical regulator of its caretaker properties. However, it is not presently known whether and how the transcription and/or DNA repair activities of BRCA1 are specifically modulated in response to DNA damage. We hypothesized that DNA damage-induced site-specific phosphorylation of BRCA1 regulates its transcription and/or DNA double-strand break repair activities. To provide support for this hypothesis, we proposed first to identify ionizing radiation-induced site-specifically phosphorylated residues on BRCA1 in complex with transcription or DNA double-strand break repair activities, and second, to determine the functional consequence of ionizing radiation-induced site-specific phosphorylation on the transcription and DNA double-strand break repair activities of BRCA1. Toward this objective, our research plan encompassed two major objectives. Frist, we proposed to biochemically purify from human cells, both prior to and following irradiation, distinct BRCA1-containing multiprotein complexes corresponding to the RNA polymerase II holoenzyme and the DNA double-strand break (DSB) repair complex containing the products of the Rad50, Mre11, and NBS1 (Nijmegan breakage syndrome) genes (Rad50/Mre11/NBS1 complex). Second, we proposed to effect direct comparative analyses of wild type BRCA1 and mutant derivatives bearing substitutions at ionizing radiation-targeted residues for their respective abilities to function in BRCA1-dependent transcription and DNA double-strand break repair assays *in vivo.*

BODY

Research Accomplishments:

Technical Objective 1. To identify ionizing radiation (IR)-induced site-specifically phosphorylated residues on BRCA1 in complex with either the RNA polymerase II holoenzyme or the Rad50/Mre11/NBS1 DNA double-strand break repair complex.

Task 1: ***Months 1-9:*** To purify distinct BRCA1-containing complexes corresponding to the RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 DNA double-strand break repair complex both prior to and following IR.

In year one, we achieved the biochemical purification of distinct BRCA1-containing multiprotein complexes implicated in transcription and DNA repair, thereby completing a significant portion of Task 1 of Technical Objective 1. The results of these studies, documented in the first year annual summary statement, were incorporated into a manuscript that has been resubmitted for publication following additional experiments, and which we include as an appendix to this annual summary statement (Please Refer to Appendix 1 – Manuscript Preprint). Using the purification strategy outlined in the appended manuscript preprint, we have succeeded in purifying distinct BRCA1-containing transcription and DNA repair complexes from HeLa S3 cells following exposure to ionizing radiation, thus completing Task 1 of Technical Objective 1.

In year one, we also identified a novel function for BRCA1 in suppressing the ligand-independent transcriptional activity of the estrogen receptor α (ERα), a principal determinant of the growth and differentiation of breasts and ovaries. This observation arose peripherally as a result of the characterization of BRCA1-containing transcription complexes, and the identification of activities previously linked to ERα. Importantly, we documented that clinically validated BRCA1 missense mutations abrogate this repression activity, thereby suggesting that its ERα-specific repression function is important for the biological activity of BRCA1 in breast and ovarian tumor suppression. Our results revealed BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded ERα, and suggested a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of breast epithelial cell proliferation. These studies offer possible insight into the tissue-specific tumor suppressor function of BRCA1 and could suggest defined molecular targets

for future intervention in breast cancer. The results of these findings were published in the *Proceedings of the National Academy of Sciences U.S.A* (2001, Vol. **98**: 9587-9592; Please refer to Appendix 2 – Manuscript Reprint) (1), and were also documented in the first year annual summary statement.

During the course of recent efforts to isolate ligand-independent ERα-associated proteins, we identified the product of the deleted in breast cancer 1 gene, DBC-1. The gene encoding DBC-1 was originally cloned from a homozygously deleted region in breast cancers on chromosome 8p21 (3). Refined analysis of breakpoints in this region, however, revealed a second gene, deleted in breast cancer 2, to encode a likely tumor suppressor, and further confirmed that DBC-1 expression is not substantially extinguished in breast or other cancers (3). We have also verified that DBC-1 is expressed in a range of breast cancer cell lines, suggesting that DBC-1 is present and presumably active in most breast cancer cells (data not shown). Recently, DBC-1 was shown to be targeted for caspase-dependent cleavage at an early point during apoptosis induced by diverse stimuli, including TNF-α, resulting in the cytoplasmic accumulation of carboxyl-terminal DBC-1 fragments that elicit mitochondrial clustering, mitochondrial matrix condensation, and sensitization to TNF-α-mediated cell death (4). Thus, caspase-dependent processing appears to unmask a propapoptotic function for the DBC-1 carboxyl-terminus that facilitates the orderly process of cell death. However, full-length DBC-1 is localized to the nucleus in healthy cells and its biological function therein has heretofore remained unknown (4). Based on our identification of DBC-1 as a ligand-independent ERα-interacting protein as well as its provocative expression profile in breast cancers, we therefore undertook to explore the physical basis, biological regulation, and functional consequence of the interaction between DBC-1 and ERα in human breast cancer cells. Below, we summarize our preliminary and recently published findings in this regard.

First, the DBC-1 amino terminus binds directly to the ERα hormone-binding domain both *in vitro* and in human breast cancer cells in a strict ligand-independent manner. Second, like E2, the antiestrogens tamoxifen and ICI 182,780 disrupt the DBC-1/ERα interaction in human breast cancer cells, thus revealing the DBC-1/ERα interface to be an unanticipated target of endocrine compounds commonly used in hormonal therapy. Third, DBC-1 depletion reduces the steady-state level of unliganded, but not liganded, ERα protein. Fourth, DBC-1 depletion inhibits estrogen-independent proliferation and promotes estrogen-independentapoptosis of ERα-positive, but not ERα-negative, breast cancer cells in a manner reversible by endocrine agents that either disrupt the DBC-1/ERα complex (E2) or that reduce the level of ERα (ICI 182,780). Together, these findings establish a principal biological function for DBC-1 in the modulation of ERα expression and hormone-independent breast cancer cell survival. The results of these findings were published in *Molecular Endocrinology* (2007, Vol. **21**: 1526-1536; Please refer to Appendix 3 - Manuscript Reprint).

Task 2: ***Months 3-18:*** To identify IR-induced site-specifically phosphorylated residues on BRCA1 present in purified RNA polymerase II holoenzyme and Rad50/Mre11/NBS1 DNA double-strand break repair complexes by both mass spectrometric analyses and immunoblot analyses using phosphopeptide-specific antibodies.

We engaged in efforts to identify IR-induced site-specifically phosphorylated residues on BRCA1 present in purified RNA polymerase II holoenzyme and Rad50/Mre11/NBS1 DNA double-strand break repair complexes by immunoblot analysis using commercially available phosphopeptide-specific antibodies and also by mass spectrometric analyses. However, we encountered significant obstacles in the identification of IR-induced site-specifically phosphorylated residues by mass spectrometric-based approaches. The difficulties encountered in this regard most likely derive from the fact that a relatively small fraction of the total cellular pool of BRCA1 is phosphorylated in response to IR. Consequently, the quantity of phosphorylated target protein purified was likely below the sensitivity limits of our instruments. Although we attempted to obtain additional starting material for these experiments., we simply ran out of time and monetory resources to complete this task.

Technical Objective 2. To determine the functional consequence of individual IR-induced site-specific phosphorylation events on the DNA double-strand break repair and transcription activities of BRCA1.

Task 1: ***Months 9-36:*** To determine the effects of targeted mutations at identified (or predicted) sites of IR-induced phosphorylation within BRCA1 on its ability to effect DNA double-strand break repair by homologous recombination and nonhomologous end-joining following its ectopic expression in Brca1-deficient cells.

As a first approach toward this objective, we initiated the functional characterization of the BRCA1/Rad50/Mre11/NBS1 DNA double-strand break repair complex purified in Task 1 of Technical Objective 1. In so doing, we made the novel discovery that BRCA1, in complex with Rad50/Mre11/NBS1, plays a critical role in the nonhomologous end-joining pathway of DNA double-strand break repair. This observation has significant implications for the role of BRCA1 in the maintenance of genomic integrity. This study was published in manuscript form in *Cancer Research* (2002, Vol. **62**: 3966-3970; Please refer to Appendix 4 – Manuscript Reprint) (2), and was also documented in the second year midterm summary statement.

Task 2: ***Months 6-24:*** To determine the effects of targeted mutations at identified (or predicted) sites of IR-induced phosphorylation within BRCA1 on its ability to control transcription following its ectopic expression in brca1-deficient cells.

As a first approach toward this objective, we established a BRCA1-dependent transcription-based assay to evaluate the effects of IR-induced site-specifically phosphorylated residues on BRCA1 identified through Task 2 of Technical Objective 1. This assay is based on the functional interaction between BRCA1 and the sequence-specific transcriptional repressor protein ZBRK1. Previously, we showed that BRCA1 is a co-repressor of ZBRK1, a sequence-specific DNA-binding transcriptional repressor of the DNA damage-inducible *GADD45* gene that functions in G2/M cell cycle checkpoint control (3). In addition to *GADD45,* potential ZBRK1 binding sites have been identified in other DNA damage-inducible genes, indicating a prospective global role for ZBRK1 and BRCA1 in the coordinate regulation of DNA damage-response genes (3). Based on these previous observations, we have proposed a model whereby ZBRK1 and BRCA1 coordinately repress a group of DNA damage response genes in the absence of genotoxic stress and, further, that DNA damage-induced cell signals relieve this repression, thereby permitting DNA damage-induced activation of these genes.

The DNA damage-induced cell signals that relieve coordinate repression of DNA damage response genes by ZBRK1 and BRCA1 is likely to involve phosphorylation. In fact, previous studies have revealed that IR-induced protein phosphorylation is required to relieve BRCA1-mediated repression of the *GADD45* gene (4). To facilitate studies designed to determine the effects of targeted mutations at identified sites of IR-induced phosphorylation within BRCA1 on its ability to control transcription, we established a BRCA1-dependent ZBRK1 transcriptional repression assay. This assay is designed to assess the BRCA1-dependent repression function of ZBRK1 in mammalian cells from a reporter template bearing ZBRK1 DNA-binding sites. To establish the utility of this system as a means to study BRCA1-dependent ZBRK1 repression, we used this system as a functional readout during experiments designed to functionally dissect ZBRK1. This study revealed that ZBRK1 harbors dual specificity zinc fingers with twin roles in DNA-binding and BRCA1-dependent transcriptional repression. Furthermore, this study provided novel insight into the mechanistic basis by which BRCA1 mediates sequence-specific control of DNA damage-responsive gene transcription by the identification of a novel BRCA1-dependent transcriptional repression domain within the ZBRK1 C-terminus. This C-terminal repression domain (CTRD) functions in a BRCA1-dependent, histone deacetylase-dependent, and promoter-specific manner and is thus functionally distinguishable from the N-terminal KRAB repression domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity. This study was published in manuscript form in *The Journal of Biological Chemistry* (2004, Vol. **279**: 6576-6587; Please refer to Appendix 5 – Manuscript Reprint) (5), and was also documented in our second year annual summary statement.

We further exploited this BRCA1-dependent ZBRK1 transcriptional repression assay to characterize novel BRCA1-associated co-repressors of ZBRK1. More specifically, during an unbiased search for novel interaction partners and possible co-regulators of the CTRD, we identified ZBRK1 itself, suggesting that

ZBRK1 can oligomerize through its CTRD. Protein interaction analyses using wild-type and mutant ZBRK1 derivatives confirmed that ZBRK1 can homo-oligomerize both *in vitro* and *in vivo*, and further mapped the ZBRK1 oligomerization domain to the CTRD C-terminus. Biochemical analyses, including protein cross-linking and gel filtration chromatography, revealed that ZBRK1 homo-oligomers exist as tetramers in solution. Functionally, we exploited the aforementioned BRCA1-dependent ZBRK1 transcriptional repression assay to show that ZBRK1 oligomerization facilitates ZBRK1-directed transcriptional repression through ZBRK1 response elements (ZREs); requirements for oligomerization-dependent repression include the ZBRK1 CTRD and KRAB repression domains, but not the DNA-binding activity of ZBRK1. These observations suggest that higher order oligomers of ZBRK1 may assemble on target ZREs through both protein-DNA and CTRD-dependent protein-protein interactions. These findings thus reveal an unanticipated dual function for ZBRK1 in both DNA-binding-dependent and –independent modes of transcriptional repression, and further establish the CTRD as a novel protein interaction surface responsible for directing homo- and heterotypic interactions necessary for ZBRK1-directed transcriptional repression. This study was published in manuscript form in the *The Journal of Biological Chemistry* (2004, Vol. **279**: 55153-55160; Please refer to Appendix 6 – Manuscript Reprint) (6).

Key Research Accomplishments.

- Biochemical resolution of distinct BRCA1-containing multiprotein complexes implicated in transcription and DNA repair.
- Novel discovery that BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor α (ERα).
- Novel discovery that BRCA1, in complex with RAD50/Mre11/NBS1, promotes nonhomologous end-joining of DNA double-strand breaks.
- Establishment of a BRCA1-dependent nonhomologus end-joining assay that will expedite studies designed to test the hypothesis that IR-induced site-specific phosphorylation of BRCA1 modulates its DNA double-strand break repair activities.
- Novel discovery within ZBRK1 of functionally bipartite zinc fingers with dual roles in sequence-specific DNA-binding and BRCA1-dependent transcriptional repression.
- Establishment of a BRCA1-dependent ZBRK1 transcriptional repression assay that will expedite studies designed to test the hypothesis that IR-induced site-specific phosphorylation of BRCA1 modulates its transcriptional regulatory activities.
- Novel discovery that ZBRK1, through its ability to homo-oligomerize, can function as both a sequence-specific DNA-binding transcriptional repressor and a DNA-binding independent transcriptional co-repressor.
- Discovery that the BRCA1-dependent ZBRK1 C-terminal repression domain (CTRD) is a novel protein interaction surface responsible for directing both homotypic and heterotypic interactions necessary for BRCA1-dependent ZBRK1 transcriptional repression.
- Novel discovery that DBC-1 is a principal determinant of steady-state ERα protein levels and survival function in human breast and ovarian cancer cells.
- Generation of murine polyconal DBC-1-specific antisera.

- Novel discovery that the DBC-1/ERα interface is a heretofore-unrecognized target of endocrine compounds (tamoxifen, ICI 182,780) currently used in breast cancer therapy.

Training Accomplishments:

Dr. Wei Tan, who previously worked on this project and authored two manuscripts in the process (please see Appendices 5 and 6– manuscript reprints) recently completed his Ph.D. thesis and graduated from the UTHSCSA/ Program in Molecular Medicine in May of 2005. Dr. Tan is currently pursuing his postdoctoral studies in the laboratory of Dr. Michael Karin in the Department of Pharmacology at the University of California, San Diego School of Medicine.

Dr. Amy M. Trauernicht, who was working to biochemically fractionate BRCA1-containing transcription and DNA repair complexes from IR-treated cells using the purification strategy developed to achieve Task 1 of Technical Objective 1 and detailed in Appendix 1, was also repsonsible for work pertaining to the identification and characterization of DBC-1 and its role in the regulation of ER expression and survival funcion in breast cancer cells. Dr. Trauernicht authored a manuscript on DBC-1 as well as an invited review (please see Appendices 3 and 9 – manuscript reprints). Dr. Trauernicht is currently pursuing her postdoctoral studies in the laboratory of Dr. Ann MacLaren in the Department of Tumor Biology at Biogen Idec, Inc. in San Diego, California.

REPORTABLE OUTCOMES TO DATE *(Year 4 reportable outcomes underlined)*.

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Awards:

1. IDEA Award DAMD17-03-1-0272, U.S. Army Dept. of Defense, BCRP, 2003.

CONCLUSIONS

We succeeded in the biochemical resolution of distinct BRCA1-containing multiprotein complexes implicated in transcription and DNA repair. We made the novel discovery that BRCA1 mediates ligand-indpendent transcriptional repression of the estrogen receptor α; this finding suggests a possible means by which BRCA1 might control breast epithelial cell proliferation, and by implication cancer risk in the breast. We identified a principal biological function for DBC-1 in the modulation of human ERα expression and survival function in breast cancer cells. We also made the novel discovery that BRCA1, in complex with Rad50/Mre11/NBS1, promotes nonhomologous end-joining of DNA double-strand breaks. This observation has significant implications for the function of BRCA1 in tumor suppression through its role in the maintenance of genomic integrity. In addition to this novel finding, we also established an efficient and reliable BRCA1-dependent DNA repair- and transcription-based functional assays, the latter of which has permitted us to make the additional novel discoveries that: (1) ZBRK1, a BRCA1-dependent transcriptional repressor, harbors zinc fingers with dual roles in sequence-specific DNA-binding and BRCA1-dependent transcriptional repression, (2) ZBRK1, through its ability to homo-oligomerize, functions dually in both DNA-binding dependent and –independent modes of transcriptional repression, and (3) the BRCA1-dependent ZBRK1 C-terminal repression domain (CTRD) is a novel protein interaction surface responsible for directing homotypic as well as heterotypic interactions necessary for ZBRK1-directed transcriptional repression. These

findings shed new light on the mechanistic basis by which BRCA1 mediates sequence-specific control of DNA damage-responsive gene transcription.

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Appendix 1

Biochemical Resolution of Distinct BRCA1-Containing Multiprotein Complexes Implicated in Transcription and DNA Repair

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Running Title: BRCA1 complexes implicated in transcription and DNA repair

BRCA1, a hereditary breast- and ovarian-specific tumor suppressor, functions in the maintenance of genome integrity and has been implicated in a diverse range of cellular processes including transcription regulation and DNA repair. However, the physical and functional relationship between BRCA1-containing activities involved in these processes remains to be fully deciphered. Here, we report the biochemical resolution of distinct multiprotein complexes comprised of BRCA1 in association with transcription and DNA repair activities. One complex, consisting of BRCA1, NBS1, Rad50, RNA polymerase II, and RNA polymerase II Mediator proteins could be resolved from a second complex comprised of BRCA1, NBS1, Rad50, Mre11, and additional polypeptides. These findings provide biochemical evidence for stable and distinct BRCA1-containing complexes with potential roles in transcription and DNA repair and, furthermore, provide evidence for an interaction of NBS1 and Rad50 with the RNA polymerase II holoenzyme. The presence of BRCA1, NBS1, and Rad50 in distinct complexes raises the possibility that these proteins represent a common core through which transcription and repair activities may be physically and functionally linked.

INTRODUCTION

Hereditary predisposition to early onset breast and ovarian cancer derives principally from germ-line mutations in either of two **BR**east **CA**ncer susceptibility genes, ***BRCA1*** and ***BRCA2*** (1,2). Considerable evidence supports the notion that *BRCA1* is a "caretaker" gene whose encoded product, a 220 kDa nuclear phosphoprotein, functions in the maintenance of

global genome stability (3-6). While the precise biochemical basis for its proposed caretaker function remains unknown, BRCA1 has nonetheless been implicated in both the regulation of transcription and the repair of damaged DNA.

Several lines of evidence support a direct role for BRCA1 in transcription control. First, the carboxyl-terminus of BRCA1 exhibits an inherent transactivation function sensitive to cancer-predisposing mutations (7-9). Second, BRCA1 has been identified as a component of the RNA polymerase II holoenzyme (10). Third, BRCA1 has been reported to interact with a variety of transcriptional activator and/or repressor proteins (11). Finally, BRCA1 activates transcription of genes that encode activities involved in DNA damage-induced cell cycle arrest and/or apoptosis. These include the cyclin-dependent kinase inhibitor *p21* and the **G**rowth **A**rrest and **D**NA **D**amage-inducible **45** (***GADD45***) genes that function in G_1/S and/or G_2/M checkpoint control, and the *bax* gene that functions in DNA damage-induced apoptosis (12-15). Collectively, these observations imply a role for BRCA1 in mediation of DNA damage-induced cell cycle arrest and/or apoptosis through control of gene transcription.

A significant body of experimental evidence also implicates BRCA1 in DNA damage repair. First, BRCA1 is known to undergo alterations in its phosphorylation status and subcellular localization in response to DNA damage (16). Second, *brca1*-deficient mouse embryonic cells are defective in the repair of both oxidative DNA damage by transcription-coupled processes and chromosomal double-strand breaks by homologous and nonhomologous recombination (17,18; Zhong, Q, Boyer, T., Chen, C.-F., Chen, P.-L., and Lee, W.-H. Manuscript submitted). Finally, BRCA1 interacts physically and functionally with the Rad50/Mre11/NBS1 protein complex that participates directly in the repair of DNA double-strand breaks (19).

Thus, while BRCA1 likely participates in the control of transcription and DNA double-strand break repair, at least in part, by virtue of its association with the RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 complex, respectively, the precise physical and functional relationship between these two sets of interacting proteins remains to be fully defined. With this issue in mind, we have undertaken the biochemical fractionation of human cell extracts to begin to decipher the protein networks through which BRCA1 functions. Our findings demonstrate that BRCA1, together with Rad50 and NBS1, can be isolated in distinct multiprotein complexes characterized by the stable association of these proteins with either transcription or repair activities. These results provide the first evidence for an association of Rad50 and NBS1 with the RNA polymerase II holoenzyme, and raise the possibility that these two proteins, along with BRCA1, represent a common core through which transcription and DNA repair activities may be linked within the cell.

RESULTS

Human HeLa cell nuclear extract was fractionated over Cibacron Hi-Trap Blue Sepharose using a linear gradient of KCl (0.1-1.5 M). Immunoblot analysis of the pre- and post-column extract revealed that the bulk of BRCA1, as well as Rad50, Mre11, NBS1, RNA polymerase II, and human Mediator proteins hSur2 and CDK8 (20) bound quantitatively to the Blue Sepharose matrix (Fig. 1; data not shown). Immunoblot analysis of individual chromatographic fractions revealed a broad elution profile for BRCA1, Rad50, and NBS1 (Fig. 1; data not shown). By contrast, hSur2 eluted early in the gradient, peaking at ~0.45 M KCl, while Mre11 eluted later, peaking at ~1.2 M KCl.

The presence of BRCA1, Rad50, and NBS1 in distinct chromatographic fractions corresponding to the peaks of a Mediator subunit on one hand (hSur2) and a double-strand break repair protein on the other (Mre11) led us to ask whether the three former proteins could be isolated in stable association with either of the latter two proteins. To address this question, Blue Sepharose fractions corresponding to the peaks of hSur2 (Fig 1; fractions 5-7) and Mre11 (Fig. 1; fractions 13-15) were pooled separately and subjected in parallel to further fractionation first by DEAE-Sepharose anion exchange and subsequently by Superose 6 gel filtration chromatography.

Immunoblot analysis revealed co-elution of hSur2, BRCA1, Rad50, and NBS1 during DEAE-Sepharose chromatography of Blue-Sepharose fractions 5-7 (Fig. 2a, lane 1 and data not shown). Superose 6 chromatography of peak DEAE-Sepharose fractions revealed co-elution of BRCA1, Rad50, and NBS1 in one major peak within the included volume (Fig. 2a; fractions 48-51), and in a second peak which corresponds to the excluded (void) volume of the Superose 6 column (Fig. 2a; fractions 39-42). The included and excluded Superose 6 peaks of BRCA1, Rad50, and NBS1 could be distinguished by the absence or presence of additional proteins. Specifically, the included peak, which eluted well ahead of the 670 kDa thyroglobulin marker, is characterized by the additional presence of RNA polymerase II holoenzyme components, including the RNA polymerase II large subunit, RPB1, and human Mediator proteins CDK8, Cyclin C, and Med7. Significantly, no Mre11 protein could be detected in these fractions. The excluded peak, by contrast, was characterized by the presence, in addition to BRCA1, Rad50, and NBS1, of RNA polymerase II and a substoichiometric level of Mre11; however, little or no hSur2, CDK8, Cyclin C or Med7 could be detected. The presence of Mre11 within the excluded peak likely derives from trace amounts of a BRCA1, Rad50, NBS1, and Mre11-containing complex incompletely resolved in the initial Blue Sepharose fractionation step. The ability of

Superose 6 to resolve this excluded peak containing Mre11 from an included peak of BRCA1, Rad50, and NBS1 in association with RNA polymerase II holoenzyme components raised the possibility that these two peaks represent stable and distinct multiprotein assemblies.

To determine if BRCA1, Rad50, and NBS1 all reside in a stable complex with RNA polymerase II holoenzyme components, individual Superose 6 column fractions corresponding to the included peaks of BRCA1, Rad50, NBS1, and holoenzyme components (Fig. 2a; fractions 48-51) were pooled and subjected to immunoprecipitation using an RNA polymerase II large subunit (RPB1)-specific monoclonal antibody, 8WG16 (21). Immunoblot analysis revealed specific co-immunoprecipitation of BRCA1, Rad50, and NBS1 along with RNA polymerase II and Mediator proteins CDK8, Cyclin C, and Med7 (Fig. 2b). This result demonstrates that these proteins all reside in a single, large molecular-size complex, which likely corresponds to the RNA polymerase II holoenzyme.

To begin to characterize the protein complex that contains BRCA1 in association with Mre11, Blue Sepharose fractions 13-15 were pooled and applied to a DEAE-Sepharose anion exchange resin. Immunoblot analysis of DEAE fractions revealed co-elution of BRCA1, Rad50, and NBS1 along with Mre11 in a 0.3M KCl step elution (data not shown). Superose 6 chromatography of peak DEAE-Sepharose fractions revealed co-elution of BRCA1, Rad50, NBS1, and Mre11 in one peak within the included volume (Fig. 3a; fractions 47-50), and in a second peak which corresponds to the excluded (void) volume of the Superose 6 column (Fig. 3a; fractions 40-43). The included and excluded Superose 6 peaks of BRCA1, Rad50, NBS1, and Mre11 could be distinguished by the absence or presence of additional proteins. Specifically, the excluded peak is characterized by the additional presence of small amounts of ATM and Rad51, although the bulk of ATM eluted in fractions corresponding to a molecular

size of its monomeric form (Fig. 3). We consider it likely that the excluded peak represents an insoluble protein aggregate, since BRCA1, Rad50, and NBS1 exhibit a propensity to precipitate from solution into aggregates that are excluded from Superose 6 (T.G. Boyer, unpublished data). Alternatively, the excluded peak could represent either an extremely large soluble protein complex or a protein/nucleic acid complex. Because these issues have not yet been thoroughly resolved, we have pursued analysis of Superose 6 fractions corresponding to the included peak of the BRCA1, Rad50, NBS1, and Mre11 proteins.

To determine if BRCA1, Rad50, NBS1, and Mre11 all reside in a stable complex, individual Superose 6 column fractions corresponding to the included peaks of the BRCA1, Rad50, NBS1, and Mre11 proteins (Fig 3a; fractions 47-50) were subjected to immunoprecipitation using a Rad50-specific monoclonal antibody, 13B3. Immunoblot analysis revealed specific co-immunoprecipitation of BRCA1, Rad50, and NBS1 along with Mre11 and at least 10 additional polypeptides (Fig. 3b). This result demonstrates that these proteins all reside in a single, large molecular-size complex.

DISCUSSION

We have undertaken the biochemical fractionation of human cell extracts in an initial effort to decipher the protein networks involved in BRCA1 function. Previous studies have implicated this tumor suppressor in both the control of transcription and the repair of damaged DNA (11). Consistent with these proposed functional roles, biochemical and protein interaction analyses have demonstrated direct and specific interaction of BRCA1 with both transcription and DNA repair activities. For example, it has been demonstrated that BRCA1 interacts individually

with the RNA polymerase II holoenzyme, with Rad51, and with the Rad50/Mre11/NBS1 DNA double-strand break repair complex (10,19,22). Thus, it appears likely that BRCA1 participates in a diverse range of DNA transactions by virtue of its association with these specific transcription and repair complexes. However, at present, the physical and functional relationship between these protein assemblies has remained undefined. We provide biochemical evidence to suggest that these activities, while perhaps linked within the cell, may nonetheless be isolated as distinct and stable macromolecular assemblies. Minimally, the proteins common to both of these identified complexes are BRCA1, Rad50, and NBS1.

The simultaneous presence of BRCA1, Rad50, and NBS1 in distinct multiprotein complexes with apparent transcription and repair functions provides a basis for the functional, and perhaps physical, linkage of these activities within the cell. We envision two alternative possibilities for the association of these two activities. First, these transcription and repair assemblies could represent components of a larger complex within the cell that has undergone fractionation in vitro (Fig 4a). Alternatively, these complexes could represent distinct assemblies in vivo that, by virtue of shared subunits, are linked functionally (Fig. 4b). For example, a dynamic redistribution of BRCA1, Rad50, and NBS1 among transcription and repair complexes could effect global alterations in these activities sufficient to meet the immediate physiological demands of the cell. The biochemical basis for such redistribution could involve phosphorylation, a notion consistent with observed alterations in the phosphorylation status and subcellular localization of BRCA1 as a consequence of cell cycle progression or cellular DNA damage (3,16). Detailed biochemical characterization of BRCA1 in association with the transcription and repair complexes identified herein should reveal whether post-translational modification represents a determinant of its interaction properties.

Our identification of both Rad50 and NBS1 in association with RNA polymerase II and transcriptional Mediator proteins represents, to our knowledge, the first demonstration of an interaction of these proteins with the RNA polymerase II holoenzyme. This observation suggests that these proteins may, like BRCA1, be dually involved in transcription and DNA repair. The precise role of these proteins in the control of transcription remains to be defined. However, such a role would not be entirely inconsistent with the observed pleiotropic features associated with an absence of NBS1 activity in Nijmegen breakage syndrome, which include microcephaly, growth and mental retardation, chromosomal instability, immunodeficiency, and a high incidence of hematopoietic malignancy (23). While NBS1 has, apart from its direct role in repair, been implicated in checkpoint control through regulation of ionizing radiation-induced p53 protein levels (24,25), a more direct role for NBS1 in control of gene transcription cannot be ruled out at present. Future analyses should serve to clarify whether and how NBS1 functions in association with the RNA polymerase II machinery to effect alterations in gene-specific transcription.

MATERIALS AND METHODS

Protein Purification - HeLa cell nuclear extract (~725 mg) was applied to Cibacron Hi-Trap Blue Sepharose (Amersham Pharmacia) at a concentration of 4.5 mg/ml protein (total of 7 X 5 ml columns; 103.5 mg protein/column) in 0.1M KCl D buffer (20). Columns were washed with four column volumes of 0.1M KCl D buffer and bound proteins subsequently eluted with a linear gradient of 0.1-1.5M KCl in D buffer over a total volume of 40 ml. Blue Sepharose fractions containing the peaks of hSur2 (fractions 5-7) and Mrre11 (fractions 13-15) as determined by

immunoblot analysis were pooled separately, dialyzed into 0.1M KCl D buffer, and processed in parallel as follows. Dialyzed Blue Sepharose fractions were applied to DEAE-Sepharose (10 mg protein/ml of resin) in 0.1M KCl D buffer. Columns were washed with four column volumes of 0.1M KCl D buffer and step-eluted with 0.3M KCl D buffer. Individual DEAE-Sepharose fractions containing the peaks of BRCA1, Rad50, and NBS1 along with either hSur2 or Mre11 proteins as determined by immunoblot analysis were pooled to a final concentration of 4 mg/ml and subjected to Superose 6 gel filtration chromatography (2 ml per 16 X 500 mm column). Individual Superose 6 column fractions were analyzed by immunoblot analysis and fractions corresponding to selected peaks as indicated were pooled, concentrated on phosphocellulose P-11 using a 0.6M KCl step elution, and subjected to immunoprecipitation analyses.

Antibody Immunoprecipitation - Monoclonal antibodies specific for the RNA polymerase II large subunit CTD (8WG16; ref. 21), human Rad50 (13B3; ref. 19), human p53 (PAb421; ref. 26), and Glutathione S-Transferase (8G11; ref. 19) were individually covalently coupled to protein G-Sepharose using dimethlylpimelimidate (27). Superose 6 column fractions containing peaks of BRCA1, Rad50, RNA polymerase II, and Mediator proteins were pooled and incubated in parallel with either 8WG16 (specific) or PAb421 (non-specific control) antibody columns in 0.3 M KCl (1/2) D buffer [(1/2) D buffer is 20 mM HEPES, pH 7.9; 0.2 mM EDTA; 10% glycerol; 5 mM β-mercaptoethanol] for 6 hours at 4° C. Superose 6 column fractions containing peaks of BRCA1, Rad50, NBS1, and Mre11 were pooled and incubated in parallel with either 13B3 (specific) or 8G11 (non-specific control) antibody columns in 0.3 M KCl (1/2) D buffer for 6 hours at hours 4° C. Column matrices were washed three times with ten column volumes of 0.3 M KCl (1/2) D buffer, once with ten column volumes of 0.1M KCl (1/2) D buffer, and eluted

with one column volume of 0.1 M glycine (pH 2.0). Column eluates were neutralized, subjected to SDS-10%PAGE, and characterized by silver stain or immunoblot analysis as indicated.

ACKNOWLEDGEMENTS

We thank P.R. Yew, P. Sung, and N. Ting for advice and comments. This work was supported by NIH grants P01CA30195 and P01CA81020 and the McDermott Endowment fund (W.-H.L.), as well as Department of Defense Grants DAMD 17-01-1-0408, DAMD 17-02-1-0584, and NIH/NCI Grant CA098301-01 (T.G.B.).

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FIGURE LEGENDS

Figure 1. BRCA1 and Rad50 co-elute from Cibacron Blue Sepharose with both transcriptional Mediator and DNA repair proteins. HeLa nuclear extract was applied to a Hi-Trap Blue column at 0.1M KCl, and bound proteins were eluted with a linear gradient of 0.1-1.5M KCl. Aliquots of the on-put nuclear extract (NEXT), the column flow-through (BS FT), and individual chromatographic fractions (numbered) were analyzed by immunoblot analysis using antibodies specific for the proteins indicated on the left of the blot. Fractions corresponding to the peaks of hSur2 (fractions 5-7) and Mre11 (fractions 13-15) were pooled separately and processed in parallel for further chromatographic analyses as indicated.

Figure 2. BRCA1, Rad50, and NBS1 reside in stable association with RNA polymerase II holoenzyme components. a. Superose 6 gel filtration profile of Blue Sepharose and DEAE-Sepharose fractionated proteins. Pooled Blue Sepharose fractions 5-7 (from Fig.1) were applied to DEAE-Sepharose and peak fractions from a 0.3M KCl step elution containing BRCA1, Rad50, and NBS1 were pooled and subjected to gel filtration on Superose 6. Aliquots of the on-

put DEAE fraction (Load) and individual column fractions (numbered) were analyzed by immunoblot analysis using antibodies specific for the proteins indicated on the left. Downward-pointing arrows indicate the positions of marker protein peaks. Fractions corresponding to the excluded (void) and included volume of the Superose 6 column are indicated. **b.** Co-immunoprecipitation of BRCA1 and NBS1 with RNA polymerase II holoenzyme components. Superose 6 fractions 48-51 (from **a**) were pooled, concentrated on phosphocellulose using a 0.6M KCl step elution (lanes 2 and 5) and subjected to immunoprecipitation with an RNA polymerase II large subunit (RPB1)-specific monoclonal antibody 8WG16 (lanes 4 and 7), or a p53-specific monoclonal antibody PAb421 (lanes 3 and 6) as a negative control. Immunoprecipitated proteins were eluted from covalently-coupled antibody columns with glycine, subjected to SDS-10%PAGE, and processed either by silver staining (lanes 1-4) or immunoblot analysis (lanes 5-7) with antibodies specific for the proteins indicated on the right. Molecular weight markers are indicated on the left. Lane 1 represents highly purified core RNA polymerase II, only the two largest subunits of which stained visibly on this gel.

Figure 3. BRCA1, Rad50, and NBS1 reside in stable association with Mre11 and additional polypeptides. a. Superose 6 gel filtration chromatography of Blue Sepharose and DEAE-Sepharose fractionated proteins. Pooled Blue Sepharose fractions 13-15 (from Fig. 1) were applied to DEAE-Sepharose and peak fractions from a 0.3M KCl step elution containing BRCA1, Rad50, NBS1, and Mre11 were pooled and subjected to gel filtration on Superose 6. Aliquots of individual column fractions (numbered) were analyzed by immunoblot analysis using antibodies specific for the proteins indicated on the left. Downward-pointing arrows indicate the positions of marker protein peaks. Fractions corresponding to the excluded (void)

and included volume of the Superose 6 column are indicated. **b.** Co-immunoprecipitation of BRCA1, Rad50, and Mre11 along with additional polypeptides. Superose 6 fractions 47-50 (from **a**) were pooled, concentrated on phosphocellulose using a 0.5M KCl step elution (lanes 1 and 4), and subjected to immunoprecipitation with a Rad50-specific monoclonal antibody 13B3 (lanes 2 and 6), or a GST-specific monoclonal antibody 8G11 (lanes 3 and 5) as a negative control. Immunoprecipitated proteins were eluted from covalently-coupled antibody columns with glycine, subjected to SDS-10%PAGE, and processed either by silver staining (lanes 1-3) or immunoblot analysis (lanes 4-6) with the antibodies specific for the proteins indicated on the right. Molecular weight markers are indicated on the left. The ~95 kDa protein specifically co-immunoprecipitated with anti-Rad50 antibody has been confirmed by immunoblot analysis to be NBS1, as indicated by the elongated arrow.

Figure 4. Schematic models for the association of BRCA1, Rad50, and NBS1 with transcription and DNA repair complexes. (**a**) The RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 DNA double-strand break repair complexes are physically linked *in vivo* to from a larger complex that undergoes fractionation *in vitro* to yield stable and distinct multiprotein assemblies. This model would imply other than a 1:1 stoichiometric ratio of BRCA1, Rad50, and NBS1 relative to other proteins within the larger complex. Presently, the stoichiometry of these proteins in complex with isolated transcription and repair activities has not been elucidated. (**b**) The RNA polymerase II holoenzyme and the Rad50/Mre11/NBS1 complexes exist *in vivo* as distinct and stable multiprotein assemblies that may be linked functionally by virtue of shared subunits. Shown here is a potential dynamic redistribution of BRCA1, Rad50, and NBS1 between transcription and repair complexes. Cell-cycle and/or DNA

damage-induced phosphorylation could represent the signal that specifies the association of these proteins with Mre11 and additional DNA double-strand break repair activities. The specific cell signal(s) that directs BRCA1, Rad50, and NBS1 to the RNA polymerase II holoenzyme is unknown.

Fig. 1

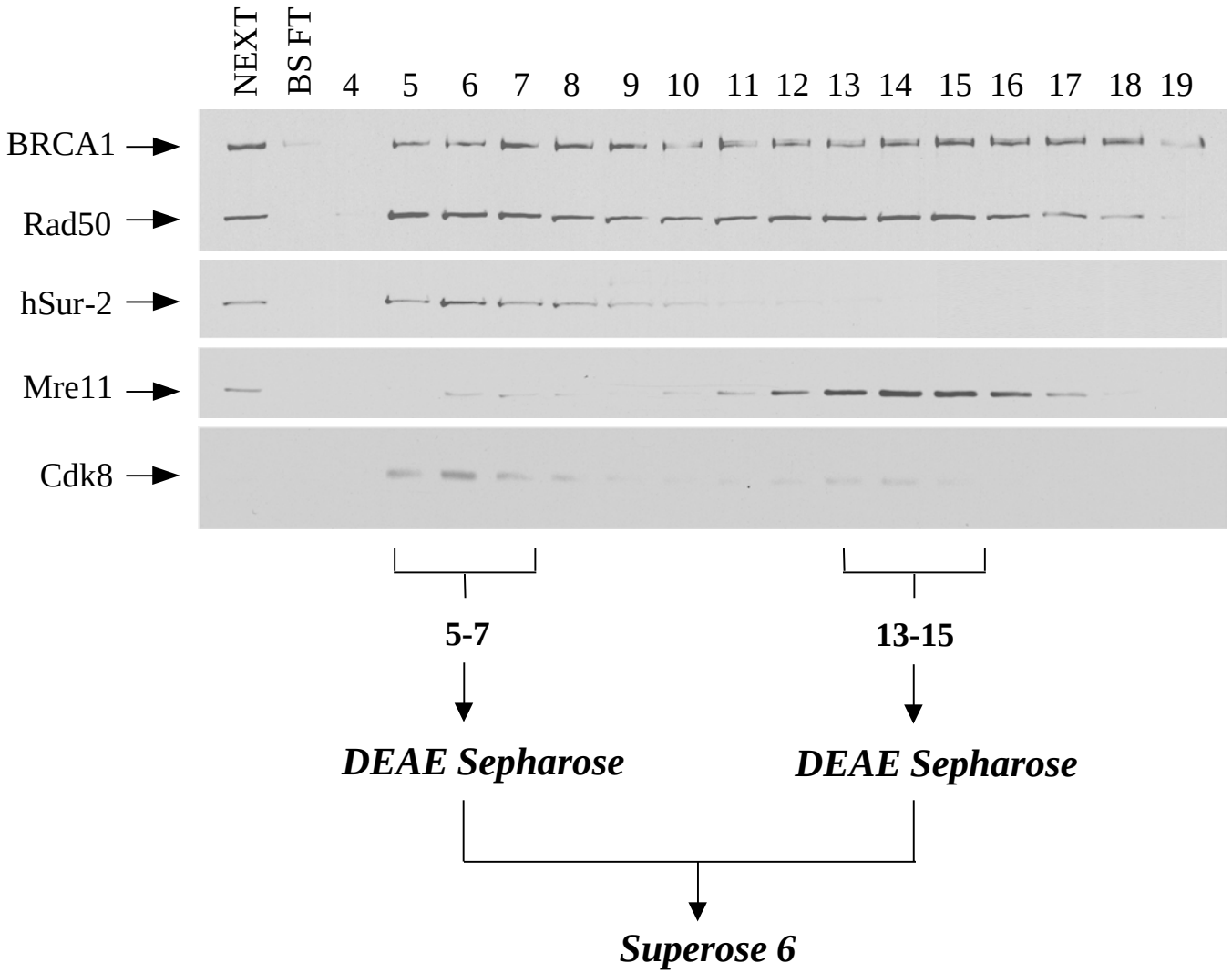
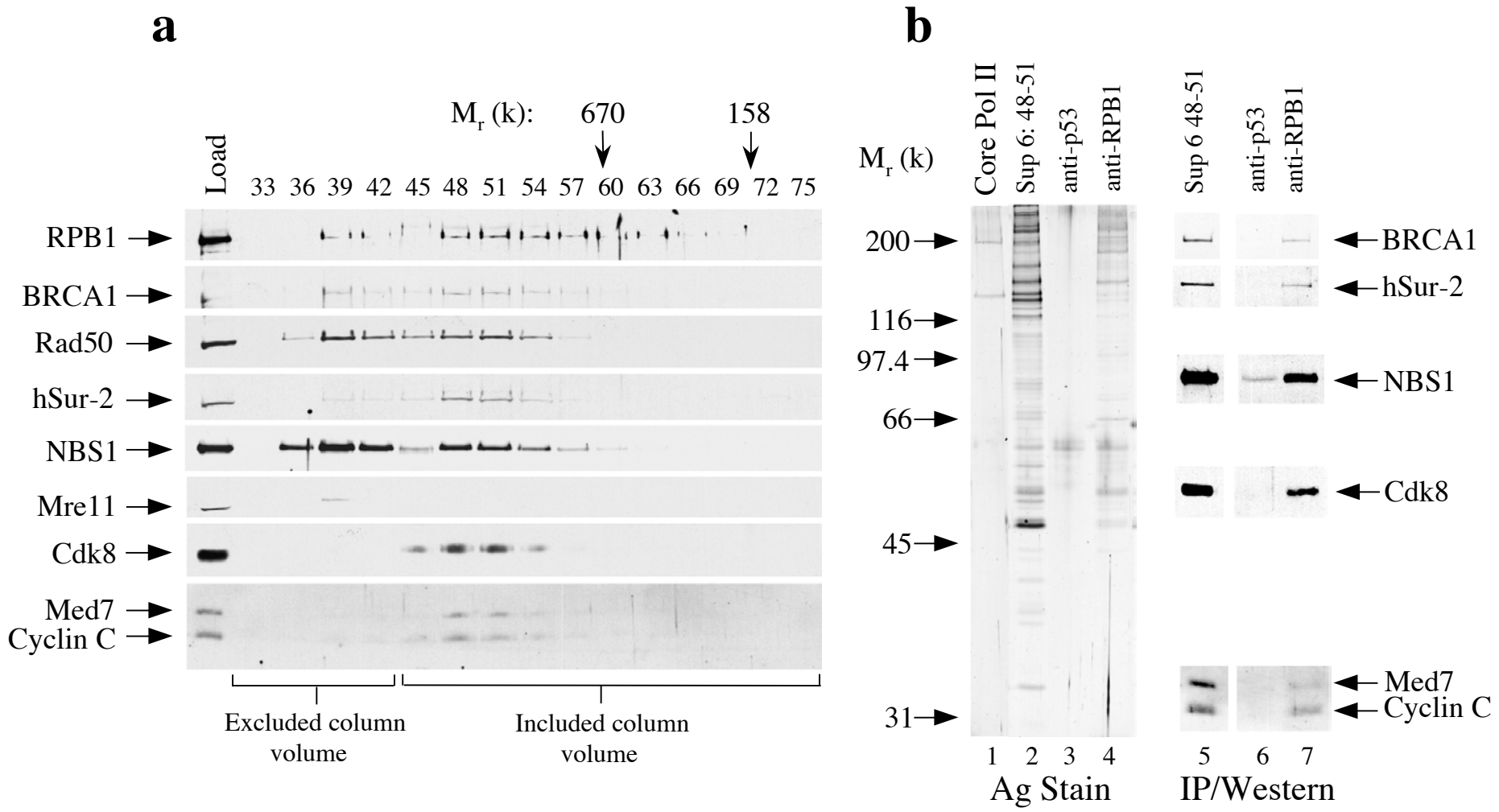

NEXT
BS FT
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
BRCA1
Rad50
hSur-2
Mre11
Cdk8
5-7
13-15
DEAE Sepharose
DEAE Sepharose
Superose 6

Fig. 2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Load
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RPB1
BRCA1
Rad50
hSur-2
NBS1
Mre11
Cdk8
Med7
Cyclin C
Excluded column volume
Included column volume
b
M$_r$ (k)
Core Pol II
Sup 6: 48-51
anti-p53
anti-RPB1
Sup 6 48-51
anti-p53
anti-RPB1
200
116
97.4
66
45
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Med7
Cyclin C
1 2 3 4
5 6 7
Ag Stain
IP/Western

Fig. 3

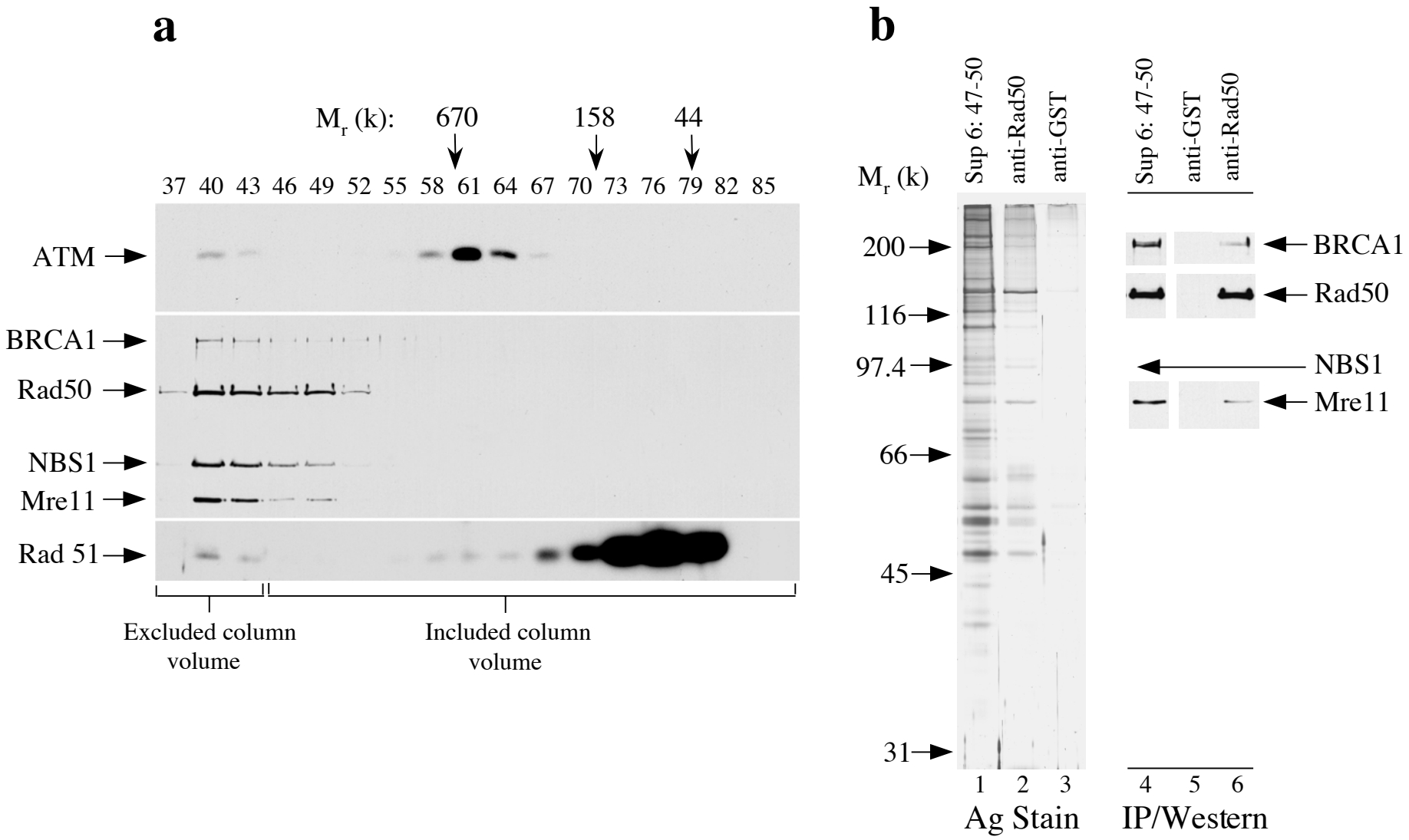
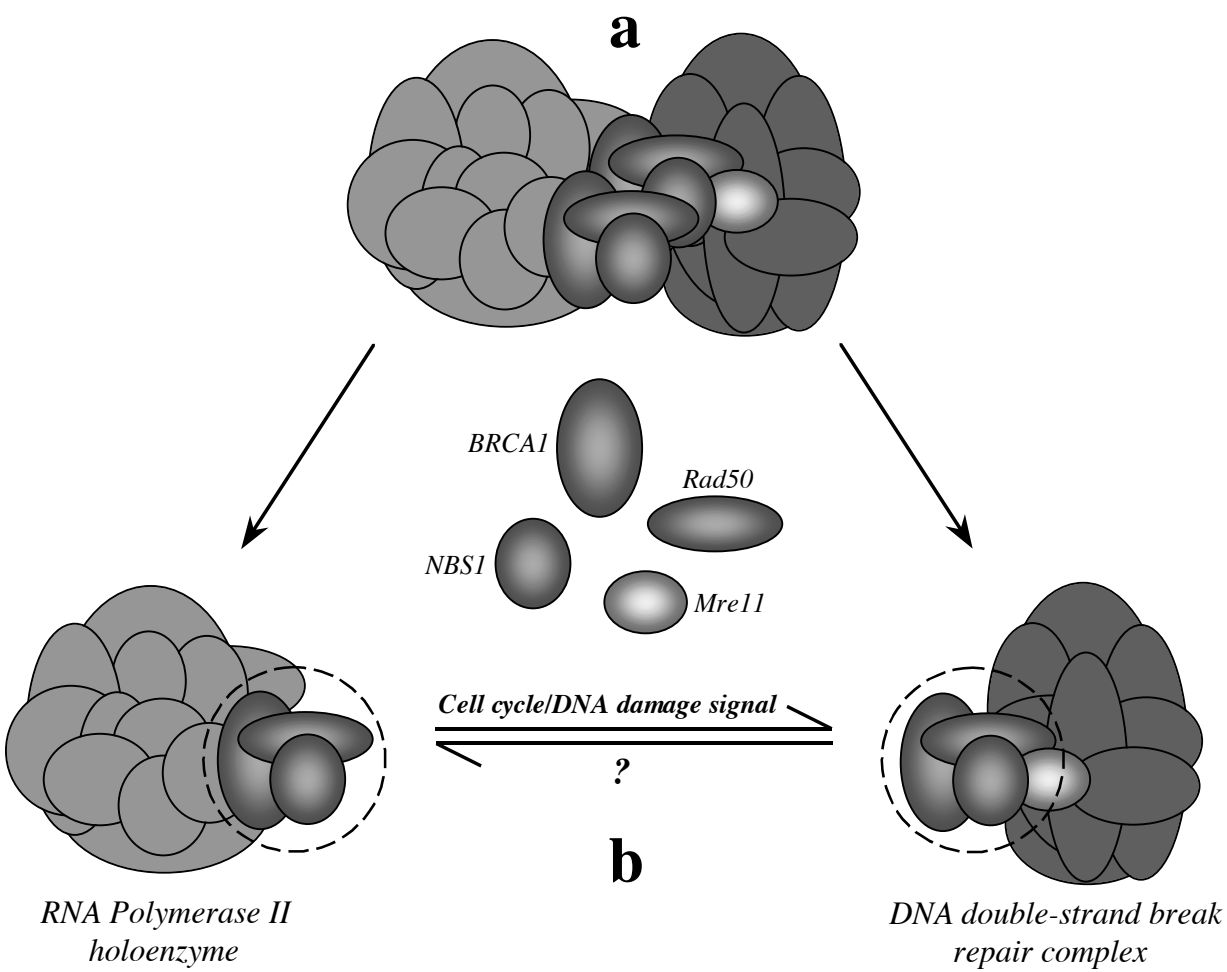

a
M$_r$ (k):
670
158
44
37 40 43 46 49 52 55 58 61 64 67 70 73 76 79 82 85
ATM
BRCA1
Rad50
NBS1
Mre11
Rad 51
Excluded column volume
Included column volume
b
M$_r$ (k)
Sup 6: 47-50
anti-Rad50
anti-GST
Sup 6: 47-50
anti-GST
anti-Rad50
200
116
97.4
66
45
31
BRCA1
Rad50
NBS1
Mre11
1 2 3
Ag Stain
4 5 6
IP/Western

Fig. 4


a
BRCA1
Rad50
NBS1
Mre11
Cell cycle/DNA damage signal
?
b
RNA Polymerase II holoenzyme
DNA double-strand break repair complex

BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor

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Edited by Robert G. Roeder, The Rockefeller University, New York, NY, and approved June 19, 2001 (received for review April 9, 2001)

Mutational inactivation of BRCA1 confers a cumulative lifetime risk of breast and ovarian cancers. However, the underlying basis for the tissue-restricted tumor-suppressive properties of BRCA1 remains poorly defined. Here we show that BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor α (ERα), a principal determinant of the growth, differentiation, and normal functional status of breasts and ovaries. In Brca1-null mouse embryo fibroblasts and BRCA1-deficient human ovarian cancer cells, ERα exhibited ligand-independent transcriptional activity that was not observed in Brca1-proficient cells. Ectopic expression in Brca1-deficient cells of wild-type BRCA1, but not clinically validated BRCA1 missense mutants, restored ligand-independent repression of ERα in a manner dependent upon apparent histone deacetylase activity. In estrogen-dependent human breast cancer cells, chromatin immunoprecipitation analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements before, but not after estrogen stimulation. Collectively, these results reveal BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα and suggest a possible mechanism by which functional inactivation of BRCA1 could promote tumorigenesis through inappropriate hormonal regulation of mammary and ovarian epithelial cell proliferation.

Germline inactivation of the gene that encodes BRCA1 represents a predisposing genetic factor in ≈15–45% of hereditary breast cancers, and minimally 80% of combined hereditary breast and ovarian cancer cases (1). Functionally, BRCA1 has been implicated in the maintenance of global genome stability (2–4), and the underlying basis for this activity likely derives from its central role in the cellular response to DNA damage, wherein it controls both DNA damage repair and the transcription of DNA damage-inducible genes (5–14).

Because the DNA damage-induced signaling pathways that converge on BRCA1 are likely to be conserved in most cell types, BRCA1 is likely to occupy a fundamental and universally conserved role in the mammalian DNA damage response. Nonetheless, germ-line inactivation of BRCA1 leads predominantly to cancer of the breast and ovary, and the underlying basis for its tissue-restricted tumor-suppressive properties thus remains undefined.

At least two hypotheses have been proposed to explain the tissue-specific nature of BRCA1-mediated tumor suppression, both of which invoke a role for estrogen in either the initiation or promotion of tumor formation (15). According to one model, the tissue-specific tumor-suppressive properties of BRCA1 derive, at least in part, from its response to tissue-specific DNA damage. In this regard, certain oxidative metabolites of estrogen itself have been documented to be genotoxic in nature (16), and BRCA1 may therefore play a role in protecting breast and ovarian tissue from estrogen-induced DNA damage.

A second model, not mutually exclusive with the one described above, to account for the this tissue-specific tumor-suppressive function invokes a role for BRCA1 in the modulation of estrogen signaling pathways and, hence, the expression of hormone-responsive genes. In this regard, BRCA1 has been reported to inhibit estrogen-dependent transactivation by the estrogen receptor α (ERα) through its direct interaction with ERα (17, 18). BRCA1 has also been reported to enhance androgen-dependent transactivation by the androgen receptor, allelic variants of which modify cancer penetrance in BRCA1 mutation carriers (19–21). Based on its postulated role in the control of nuclear hormone signaling pathways, BRCA1 could therefore influence epithelial cell proliferation and, by implication, cancer risk in tissues such as breast and ovary.

Herein, we describe a role for BRCA1 in mediating ligand-independent transcriptional repression of the ERα. Initial efforts to elucidate the mechanistic basis for this repression reveal that BRCA1 represents a ligand-reversible barrier to transcriptional activation by unliganded promoter-bound ERα. These findings suggest a potential role for BRCA1 in the proliferative control of normal estrogen-regulated tissues and a potential basis by which its mutational inactivation could promote tumorigenesis through inappropriate hormonal responses.

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Materials and Methods

Cell Culture. *p53−/−* (Brca1+/+) and *p53−/−; Brca1−/−* (Brca1−/−) mouse embryonic fibroblasts (MEFs) were cultured as described (14). Human MCF7 cells were maintained in DMEM supplemented with 10% FCS. Human BG-1-derived NEO1 and AS4 cell lines were maintained as described (22). Depletion of hormone ligands for nuclear/steroid receptor activation studies was achieved by cell culture in medium containing either 10% charcoal/dextran-treated serum (HyClone) or defined serum replacement 2 (Sigma).

Plasmids and Transfections. Transfection assays were performed by using the following conditions.

Reporter plasmids. Used at 0.5 μg each, including pTRE(F2)-TK-Luc, pGRE-TK-CAT, pERE-TK-Luc, or pPRE-TK-CAT (23); 0.5 μg of pGAL4-SV40-Luc containing five GAL4 DNA-binding sites upstream of the minimal simian virus 40 (SV40) promoter, driving expression of the luciferase reporter gene in the pGL2 vector (Promega); and 0.5 μg of pGAL4-E1B-Luc (24).

Receptor expression plasmids. Used at 1.0 μg each, including RSV-hTRβ, RSV-hGR, RSV-hERα, and RSV-hPRβ (23).

BRCA1 expression plasmids. Used at 1.0 μg each, including pcDNA3.1-BRCA1, pcDNA3.1-BRCA1-A1708E, pcDNA3.1-BRCA1-Q356R, and pcDNA3.1-BRCA1-A1708E/Q356R expressing either human wild-type BRCA1 or familial breast cancer-derived BRCA1 mutants (14).

Abbreviations: ERα, estrogen receptor α; MEF, mouse embryonic fibroblast; E2, 17β-estradiol; RT-PCR, reverse transcription–PCR; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; AF-1, N-terminal ligand-independent activation function; AF-2, C-terminal ligand-inducible activation function.

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Chimeric activators. Used at 1.0 μg of GAL4-ERα, generated by an amino-terminal fusion of ERα with the GAL4 DNA-binding domain in pM3 (25); 0.1 μg of pVP16-GAL4 or pVP16-GAL4-ERα containing ERα amino acids 251–595, as described (26).

MEFs (6×10^4) or BG-1 cells (2×10^5) cultured in ligand-free medium were transfected by Lipofectin-based methods under serum-free conditions. Culture medium was replaced with fresh ligand-free medium 24 h after transfection, and 10^{-7} M 17β-estradiol (E2) or 330 nM trichostatin A was added as indicated. Cells were harvested 48 h after transfection for luciferase assay as described (14) or chloramphenicol acetyltransferase (CAT) assay by liquid scintillation counting (Promega).

Reverse Transcription (RT)-PCR Analysis. BG-1-derived cells were cultured in ligand-free medium for at least 5 days, and treated with 10^{-7} M E2 for 1 h as indicated. Approximately 15 μg of total cellular RNA was subjected to semiquantitative RT-PCR analysis following a procedure previously described for estrogen-responsive genes (27, 28).

Chromatin Immunoprecipitation (ChIP). MCF7 cells were cultured in ligand-free medium for at least 5 days and treated with 10^{-7} E2 for 1 h as indicated. ChIP assays were performed as described (29).

Antibodies. Antibodies used for soluble and chromatin immunoprecipitations and immunoblot analyses were as follows: BRCA1 (mAb 6B4); ERα (rabbit polyclonal antibody HC-20 or mouse mAb D-12, Santa Cruz Biotechnology); CtIP (mAb 19E8); TFIIH p89 (rabbit polyclonal antibody S-19, Santa Cruz Biotechnology); glutathione *S*-transferase (MAb 8G11); RNA polymerase II large subunit (mAb 8WG16); cathepsin D (rabbit polyclonal antibody 06-467, Upstate Biotechnology, Lake Placid, NY); pS2 (mouse mAb V3030, Biomeda, Hayward, CA); human progesterone receptor β (mouse mAb PriB-30, Santa Cruz Biotechnology); p84 (mAb 5E10).

Results

BRCA1 has been shown to modulate the ligand-dependent transcriptional activity of specific members of the nuclear hormone receptor family (17–20). However, endogenous BRCA1 present in the transfected cell lines used in previous studies precluded analysis of the effect of BRCA1 on the ligand-independent function of these receptors. Therefore, to more directly assess the role of BRCA1 in nuclear receptor transactivation without competition from endogenous BRCA1, we analyzed a panel of nuclear receptors for their respective ligand-independent transcriptional activities in Brca1-nullizygous MEFs.

A set of minimal thymidine kinase (TK) promoters, each under control of distinct hormone-response elements specific for either the human thyroid receptor β (TRβ), the glucocorticoid receptor (GR), the ERα, or the progesterone receptor β (PRβ) were individually tested for their respective abilities to direct expression of a reporter gene in the absence or presence of each corresponding receptor (absent ligand) after transfection into Brca1-proficient (Brca1+/+) or Brca1-deficient (Brca1−/−) MEFs (14). Unexpectedly, we observed significant ligand-independent activation of reporter gene expression directed by both the progesterone receptor β and the ERα in Brca1-deficient MEFs compared with Brca1-proficient MEFs (Fig. 1*A*). By contrast, no ligand-independent stimulation of reporter activity directed by either the thyroid receptor β or the glucocorticoid receptor could be observed in Brca1-deficient MEFs (Fig. 1*A*). Interestingly, although E2 activated the ERα in both Brca1-proficient and Brca1-deficient MEFs, the relative level of induction observed in Brca1-deficient MEFs was diminished 2-fold relative to Brca1-proficient MEFs (Fig. 1*B*). We confirmed by immunoblot analysis that the transfected ERα was expressed equivalently in BRCA1-proficient and BRCA1-deficient MEFs, thus excluding the possibility that differences in receptor activity derive from differences in receptor protein expression (Fig. 1*C*).

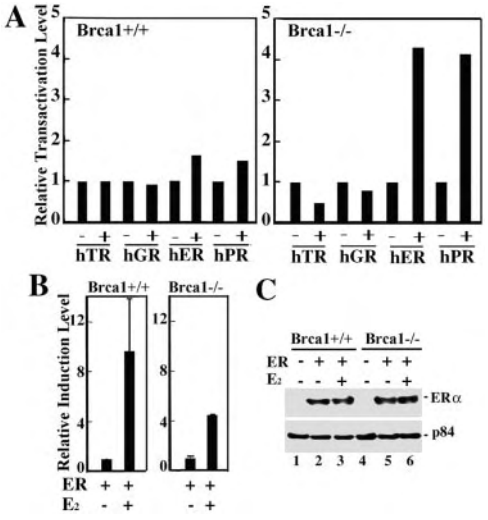

Fig. 1. BRCA1 mediates ligand-independent repression of the receptors for estrogen and progesterone. (*A*) Brca1+/+ and Brca1−/− MEFs in hormone-free media were transfected with reporter plasmids (pTK-Luc or pTK-CAT) carrying response elements specific for individual hormone receptors without (−) or with (+) plasmids expressing the human thyroid receptor β (hTR), glucocorticoid receptor (hGR), estrogen receptor α (hER), or progesterone receptor β (hPR). Transfections performed without (−) receptor expression plasmids were performed instead with a molar equivalent of the backbone expression plasmid pRSV. The relative transactivation level represents the fold-increase in transfected reporter gene activity measured in cells cotransfected with a specific receptor expression plasmid relative to the level of transfected reporter gene activity measured in cells cotransfected with the backbone pRSV expression plasmid. Reporter gene activity was first normalized to β-galactosidase activity obtained by cotransfection of an internal control pSV40-β-gal expression plasmid as described (14). Expression of the pSV40-β-gal plasmid was not affected by the absence of presence of BRCA1 or any of the nuclear hormone receptors analyzed (data not shown). (*B*) Brca1+/+ and Brca1−/− MEFs in estrogen-free media were transfected with pERE-TK-Luc carrying three copies of the consensus estrogen response element (ERE) with (+) pRSV-ERα in the absence (−) or presence (+) of E2 (10^{-7} M) before assay for luciferase activity. The relative induction level represents the relative transactivation level measured in the presence of E2 divided by the relative transactivation level measured in the absence of E2. (*C*) Brca1+/+ (lanes 1–3) and Brca1−/− (lanes 4–6) MEFs either untransfected (lanes 1 and 4) or transfected (lanes 2, 3, 5, and 6) with an ERα-expressing vector were lysed, and immunoprecipitated ERα was immunoblotted with ERα-specific antibodies (*Upper*). Immunoblot analysis of the nuclear matrix protein p84 (*Lower*) indicates that nearly equivalent amounts of each cell lysate were used in the immunoprecipitations.

Ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs repressed ligand-independent activation directed by ERα (Fig. 2*A*). Likewise, a BRCA1 derivative carrying a familial breast cancer-derived missense mutation in the ring finger (C64G) also repressed ligand-independent activation by ERα (Fig. 2*A*). By contrast, BRCA1 derivatives carrying familial breast cancer-derived missense mutations in either an exon 11-encoded region that binds Rad50 and the transcriptional repressor ZBRK1 (Q356R) or the C-terminal BRCT domain (A1708E) abolished the ability of BRCA1 to repress ligand-independent transactivation directed by ERα (Fig. 2*A*). Differences in the transcriptional repression activities of the various BRCA1 mutant derivatives could not be attributed to differences in their respective levels of expression because each of the BRCA1 mutant derivatives was expressed at a level comparable

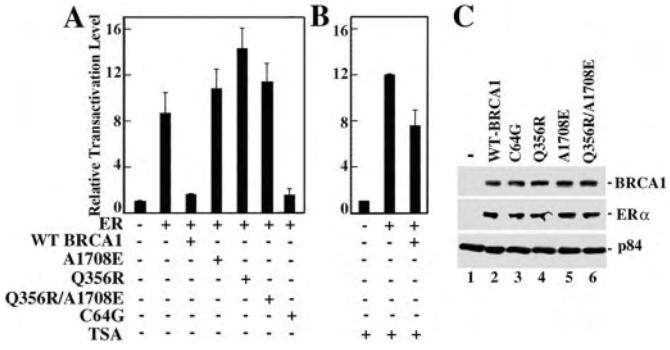

Fig. 2. Ectopic expression of wild-type BRCA1 in Brca1-deficient MEFs restores ligand-independent repression of ERα transactivation in a histone deacetylase (HDAC)-dependent manner. (*A* and *B*) Brca1$-/-$ MEFs in estrogen-free media were transfected with pERE-TK-Luc without ($-$) or with ($+$) pRSV-ERα, pCDNA3.1-BRCA1 expressing wild-type human BRCA1 (WT), or pCDNA3.1-BRCA1 derivatives bearing missense mutants A1708E, Q356R, A1708E/Q356R, or C64G before assay for luciferase activity. Where indicated, trichostatin A (TSA; 330 nM) was also included. (*C*) Brca1$-/-$ MEFs in estrogen-free media were untransfected (lane 1) or cotransfected with expression vectors for ERα and either wild-type BRCA1 (lane 2) or various BRCA1 mutant derivatives (lanes 3–6) as indicated. Cells were lysed, and immunoprecipitated BRCA1 and ERα were subjected to immunoblot analysis using antibodies specific for BRCA1 (*Top*) or ERα (*Middle*). Immunoblot analysis of the nuclear matrix protein p84 (*Bottom*) indicates that nearly equivalent amounts of each cell lysate were used in the immunoprecipitations.

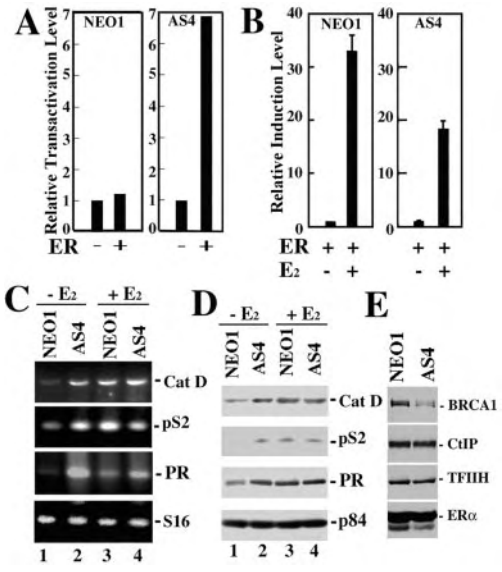

Fig. 3. Reduced BRCA1 expression in BG-1 human ovarian adenocarcinoma cells is accompanied by increases in estrogen-independent expression of estrogen-responsive genes. (*A*) Retroviral vector-infected (NEO1) and BRCA1 antisense-infected (AS4) BG-1 cell clones in estrogen-free media were transfected with pERE-TK-Luc without ($-$) or with ($+$) pRSV-ERα before assay for luciferase activity. (*B*) NEO1 and AS4 cells in estrogen-free media were transfected with pERE-TK-Luc with ($+$) pRSV-ERα in the absence ($-$) or presence ($+$) of E2 (10^{-7} M) before assay for luciferase activity. (*C*) NEO1 (lanes 1 and 3) or AS4 (lanes 2 and 4) cells in estrogen-free media were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) with E2 (10^{-7} M) for 1 h. Cells were harvested and processed for semiquantitative RT-PCR analysis using primers specific for the estrogen-responsive cathepsin D (Cat D), pS2, and progesterone receptor genes, as well as the estrogen-nonresponsive ribosomal S16 gene. (*D*) NEO1 (lanes 1 and 3) or AS4 (lanes 2 and 4) cells (5×10^6) in estrogen-free media were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) with E2 (10^{-7} M) for 24 h. Culture medium was concentrated 10-fold by using a Centriprep YM-3 device, and 1/10th of the concentrate was resolved by SDS/15%PAGE and processed for immunoblot analysis using antibodies specific for pS2. Cells were also lysed in RIPA buffer, and 1/10th of the lysate was subjected to immunoblot analysis using antibodies specific for progesterone receptor β (PR), cathepsin D (Cat D), or nuclear matrix protein p84, which served as an internal loading control. (*E*) Whole cell lysates derived from NEO1 and AS4 cells were resolved by SDS/10%PAGE and processed for immunoblot analysis using antibodies specific for BRCA1, CtIP, and the p89 subunit of the transcription factor IIH (TFIIH), the latter two of which served as internal loading controls. The ERα-positive status of these cells was verified by using an ERα-specific rabbit polyclonal antibody. Densitometric quantitation of the immunoblot and normalization to the CtIP and TFIIH signals revealed BRCA1 expression to be reduced by 70% in AS4 cells compared with NEO1 cells.

to wild-type BRCA1 (Fig. 2*C*). BRCA1-mediated, ligand-independent repression of ERα was largely reversed by trichostatin A, implicating histone deacetylase (HDAC) activity in this process (Fig. 2*B*). Collectively, these results reveal a function for BRCA1 as a repressor of ligand-independent, ERα-mediated transactivation.

To confirm these results in a biologically relevant cell type, we analyzed the ligand-independent activity of ERα in human ovarian adenocarcinoma BG-1 cells, which are ERα-positive and estrogen-dependent for growth (30). Previously, Annab *et al.* (22) described the generation of independent BG-1 clonal cell lines that support stably reduced BRCA1 mRNA and protein levels by retroviral-mediated BRCA1 antisense delivery. We tested the ability of ERα to direct ligand-independent transcription of the ERE-TK-Luc reporter gene after transfection into either a control retroviral vector-infected BG-1 clonal cell line (NEO1) or, alternatively, a BRCA1 antisense-infected BG-1 clonal cell line (AS4) exhibiting severely reduced BRCA1 expression levels (Fig. 3*E*; ref. 22). Consistent with the results obtained in MEF cells, ERα exhibited significantly increased ligand-independent activity in BRCA1-deficient AS4 cells compared with BRCA1-proficient NEO1 cells (Fig. 3*A*). We also observed a 2-fold reduction in the relative level of E2-mediated induction of reporter gene activity in AS4 cells compared with NEO1 cells, once again consistent with the results obtained in MEF cells (Fig. 3*B*). These results confirm that in a biologically relevant epithelial cell type, BRCA1 can mediate repression of ligand-independent ERα transactivation activity.

To determine whether the reduced BRCA1 expression levels in AS4 cells could be correlated with an increase in the ligand-independent expression of endogenous estrogen-responsive genes, we performed a direct comparative analysis of NEO1 and AS4 cells with respect to their ligand-independent expression of several estrogen-responsive genes. Individual monolayer cultures of NEO1 and AS4 cells were grown in the absence of estrogen for 5 days followed by the addition of either no hormone or, alternatively, E2 (10^{-7} M) for 1 h. Subsequently, cells were harvested and analyzed by semiquantitative RT-PCR for the expression levels of the endogenous estrogen-responsive pS2, cathepsin D, and progesterone receptor genes.

Relative to the expression level of an internal control ribosomal S16 gene, we observed increases in the ligand-independent expression levels of the pS2, cathepsin D, and progesterone receptor genes of 3-, 5-, and 9-fold, respectively, in BRCA1-deficient AS4 cells compared with BRCA1-proficient NEO1 cells (Fig. 3*C*). Interestingly, although the addition of E2 stimulated transcription of the pS2, cathepsin D, and the progesterone receptor genes in NEO1 cells, no such E2-dependent increase in the transcription of these genes could be observed in AS4 cells (Fig. 3*C*). Qualitatively similar results were observed at the protein level by immunoblot analysis. Relative to the level of an internal control protein (nuclear matrix protein p84), E2-independent increases in the steady-state levels of the pS2, cathepsin D, and progesterone receptor proteins could be observed in AS4 cells compared with NEO1 cells (Fig. 3*D*). Furthermore, although the addition of E2 elevated the steady-

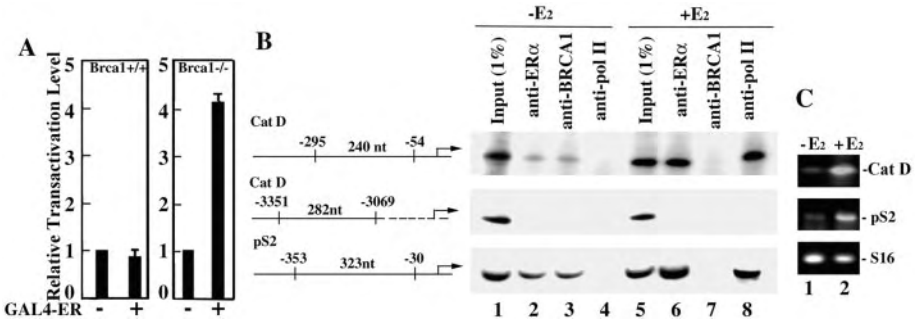

Fig. 4. BRCA1 represses unliganded promoter-bound ERα-mediated transactivation. (*A*) Brca1+/+ and Brca1−/− MEFs were transfected with a pGAL4-SV40-Luc reporter plasmid either without (−) or with (+) a pGAL4-ERα expression plasmid before assay for luciferase activity. (*B*) Schematic diagram of the cathepsin D (Cat D) and pS2 gene regions targeted for ChIP analysis. Negative numbers refer to sequence coordinates that delimit PCR amplicons defined by gene-specific primer pairs relative to the transcription initiation site (right-angled arrow). Numbered nucleotides (nt) refer to the expected sizes of PCR-amplified products. MCF-7 cells, cultured the absence of estrogen, were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h. Soluble chromatin was prepared and subjected to immunoprecipitation by using monoclonal antibodies specific for ERα (anti-ERα), BRCA1 (anti-BRCA1), or the RNA polymerase II large subunit (anti-pol II). Immunoprecipitated DNA was PCR-amplified by using primers that span the indicated regions of the cathepsin D and pS2 gene promoters. Input (1%) of the soluble chromatin subjected to immunoprecipitation was PCR-amplified directly by using each primer pair as indicated. (*C*) MCF-7 cells, cultured in the absence of estrogen, were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h before harvest and processing for semiquantitative RT-PCR analysis using primers specific for the estrogen-responsive cathepsin D (Cat D) and pS2 genes, as well as the estrogen-nonresponsive ribosomal S16 gene.

state level of each of these proteins in NEO1 cells, no such E2-dependent increase could be observed in AS4 cells (Fig. 3*D*). Quantitative differences between RT-PCR and immunoblot analyses could reflect the influence of posttranscriptional regulatory processes. Nonetheless, RT-PCR and immunoblot analyses both reveal that the ligand-independent expression of endogenous ERα-target genes is increased in BRCA1-deficient cells. Collectively, these results implicate BRCA1 in the ligand-independent repression of endogenous estrogen-responsive genes.

To explore the mechanism by which BRCA1 mediates ligand-independent repression of ERα, we first determined whether BRCA1 could interact with unliganded ERα *in vivo* by coimmunoprecipitation of the two proteins in human breast cancer MCF7 cells cultured in the absence of estrogen. Consistent with previous results (18), BRCA1 could be specifically coimmunoprecipitated with unliganded ERα, thus demonstrating that the two proteins can interact *in vivo* in a ligand-independent manner (data not shown).

To explore the possibility that BRCA1 represses the transactivation function of promoter-bound, unliganded ERα, we first tested the effect of BRCA1 on the ligand-independent transcriptional activity of ERα tethered to the yeast GAL4 DNA-binding domain by using a reporter template bearing GAL4 DNA-binding sites. This approach permitted us to assess the effect of BRCA1 on the transactivation function of unliganded ERα independent of any effects that BRCA1 might have on the DNA-binding activity of unliganded ERα. GAL4-ERα was cotransfected along with a GAL4-SV40-luciferase reporter template into Brca1-proficient and Brca1-deficient MEFs. We observed significant ligand-independent stimulation of reporter activity in Brca1-deficient, but not in Brca1-proficient, MEFs (Fig. 4*A*), suggesting one mechanism by which BRCA1 mediates ligand-independent repression of ERα is through direct repression of the DNA-bound receptor.

To confirm this observation under biologically relevant conditions *in vivo*, we used ChIP analyses to determine whether BRCA1 can be recruited directly to estrogen-responsive promoters in the absence of ligand. MCF-7 cells were grown in the absence of estrogen for 5 days followed by the addition of either no hormone or, alternatively, E2 (10^{-7} M) for 1 h. Promoter occupancy before and after E2 treatment at the estrogen response elements within the endogenous pS2 and cathepsin D gene promoters by ERα, BRCA1, and RNA polymerase II was then monitored by ChIP using antibodies specific for each of the three proteins and semiquantitative PCR with primers flanking the estrogen response elements of the pS2 and cathepsin D promoters. In the absence of E2, ERα could be detected in association with both the pS2 and cathepsin D promoters, and this level was increased dramatically by the addition of E2 (Fig. 4*B*, lanes 2 and 6). Strikingly, we also observed pS2 and cathepsin D promoter occupancy by BRCA1 in the absence of E2, and a reduction in such occupancy after E2 treatment (Fig. 4*B*, lanes 3 and 7). By contrast, RNA polymerase II could be detected only following, but not before, E2 treatment, consistent with its ligand-dependent recruitment concomitant with transcriptional activation of the pS2 and cathepsin D genes (Fig. 4*B*, lanes 4 and 8 and *C*, lanes 1 and 2). The specificity of factor association within the estrogen-responsive region of the pS2 and cathepsin D promoters was confirmed by ChIP analysis using antibodies specific for ZBRK1, a sequence-specific DNA-binding transcriptional repressor that does not bind to pS2 or cathepsin D promoter sequences (14). ZBRK1-specific antibodies failed to immunoprecipitate pS2 and cathepsin D promoter sequences (data not shown). Further specificity of the ChIP assay was demonstrated by the inability to detect occupancy by ERα, BRCA1, or RNA polymerase II of a region ≈3 kb upstream of the cathepsin D promoter (Fig. 4*B*). These results thus reveal the association of BRCA1 with unliganded ERα at endogenous estrogen-responsive promoters under physiologically relevant conditions *in vivo*.

Like other steroid receptors, ERα contains two transactivation domains, an N-terminal ligand-independent activation function (AF-1) that is targeted by a variety of steroid-independent cell-signaling pathways, and a C-terminal ligand-inducible activation function (AF-2) that resides within the receptor ligand-binding domain (31, 32). Previous analyses of ERα suggest a model whereby repressive factors binding to sequences within its C-terminal ligand-binding domain repress constitutively active AF-1 in the absence of an agonist or in the presence of an antagonist (26, 33). To determine whether ligand-independent repression of ERα by BRCA1 is mediated through the ERα ligand-binding domain, we tested the ligand-independent activity of a VP16-GAL4-ERα receptor chimera after its expression in both BRCA1-proficient and BRCA1-deficient BG-1 clonal cell lines. This chimera encodes ERα amino acids 251–595, including the hinge region and the ligand-binding domain, fused C-terminally to the hybrid transactivator VP16-GAL4 (26).

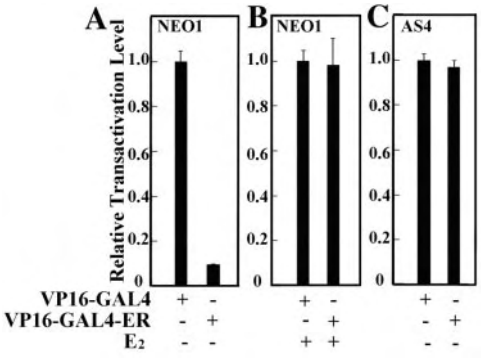

Fig. 5. VP16-GAL4-ERα exhibits hormone-dependent activity in BRCA1-proficient cells and constitutive activity in BRCA1-deficient cells. NEO1 (*A* and *B*) and AS4 (*C*) cells in estrogen-free media were transfected with a GAL4-E1B-Luc reporter plasmid along with (+) plasmids expressing either VP16-GAL4 or VP16-GAL4-ERα. Subsequently, transfected cells were either untreated (−) or treated (+) with E2 (10^{-7} M) before assay for luciferase activity.

Previously, deletion analysis of this receptor chimera revealed that constitutive VP16-GAL4-ERα activity could be recovered by the removal of sequences within the ligand-binding domain of the ERα moiety, thereby implicating the ERα ligand-binding domain in ligand-independent transcriptional repression of a neighboring constitutive activation domain (26). To determine whether this ligand-independent repression is mediated by BRCA1, we transfected the VP16-GAL4-ERα chimera along with a reporter template bearing GAL4 DNA binding sites into both BRCA1-proficient NEO1 cells and BRCA1-deficient AS4 cells. In NEO1 cells, the VP16-GAL4-ERα chimera exhibited minimal constitutive transactivation activity in the absence of E2; in response to E2, this level was dramatically increased to one approaching that of the potent VP16-GAL4 activator alone (Fig. 5 *A* and *B*). By contrast, in AS4 cells the VP16-GAL4-ERα chimera exhibited constitutive transactivation activity comparable to that exhibited by the VP16-GAL4 activator alone (Fig. 5*C*). The addition of E2 had a minimal effect on the elevated constitutive transactivation activity of the ERα chimera in AS4 cells (data not shown), suggesting that the principle effect of E2 is to override a ligand-independent barrier to the transactivation activity of the chimeric receptor. This barrier is present in NEO1 cells, but deficient in AS4 cells. Similar results were also observed by using isogenic Brca1-proficient and Brca1-deficient MEFs, eliminating the possibility that cell type-specific peculiarities contribute to the differential transactivation properties of the VP16-GAL4-ERα chimera in the presence and absence of BRCA1 (data not shown). Collectively, these results reveal the ERα ligand-binding domain to be a platform for the recruitment of BRCA1 from which the latter may confer ligand-independent repression on a linked activation domain. Hence, we conclude that BRCA1-mediated ligand-independent repression of ERα is likely to be mediated through the ERα ligand-binding domain.

Discussion

Recently, BRCA1 has been proposed to inhibit the ligand-dependent transcriptional activity of ERα through a direct interaction between the two proteins (18). Our current analysis of ERα transcriptional activity in Brca1-nullizygous MEFs revealed BRCA1 to be a ligand-reversible barrier to transcriptional activation by unliganded ERα. The biological relevance of this finding is further strengthened by the observation that BRCA1 also mediates ligand-independent repression of the ERα in human ovarian adenocarcinoma cells.

The underlying mechanism by which BRCA1 mediates ligand-independent repression of ERα transcriptional activity appears to involve targeted recruitment by unliganded, promoter-bound ERα of a BRCA1-associated HDAC activity. This conclusion is based first on the observation that the HDAC inhibitor trichostatin A can effectively reverse ligand-independent repression mediated by BRCA1 and, second, on the results of ChIP analyses, which revealed the association of unliganded ERα with BRCA1 on endogenous estrogen-response elements *in vivo*. A likely target of BRCA1-mediated ligand-independent ERα repression is the constitutive AF-1 activation domain within ERα. Previous studies have indicated that antagonist-bound AF-2 can repress AF-1 activity through the recruitment of the nuclear corepressor N-CoR (33), whereas the ligand-binding domain of unliganded ERα can repress a linked heterologous activation domain in a ligand-reversible manner, presumably by the recruitment of a soluble corepressor (26). Our observation that an estrogen-dependent VP16-GAL4 chimeric transactivator carrying the ERα ligand-binding domain exhibits constitutive activity in BRCA1-deficient, but not in BRCA1-proficient BG-1 cells, reveals the ERα ligand-binding domain to be a potential site of BRCA1 recruitment for ligand-independent repression of a linked activation domain. Hence, BRCA1 could be recruited to the ERα ligand-binding domain as part of a larger repression complex to silence AF-1 function in the absence of ligand. The recent report of a direct interaction between BRCA1 and the ERα ligand-binding domain (18) lends additional support to this model.

Should BRCA1 function to inhibit the ligand-dependent transcriptional activity of ERα (17, 18), it seems unlikely to do so through a mechanism that involves promoter-bound ERα. Our ChIP analysis revealed the association of BRCA1 with ERα at endogenous estrogen-response elements before, but not after, estrogen stimulation. Thus, we favor a model in which BRCA1, along with an associated corepressor(s) that minimally includes an HDAC activity, is recruited by unliganded, promoter-bound ERα to effectively silence the constitutive AF-1 activation domain and thereby repress estrogen-responsive target gene transcription. After estrogen stimulation, a ligand-induced conformational change within ERα could lead to enhanced affinity of the ERα for its cognate binding site and release of a BRCA1-containing repression complex, thereby liberating AF-1 and AF-2 to synergistically recruit coactivators and the RNA polymerase II holoenzyme to promote transcription (29). It is also possible that BRCA1 could function additionally as a barrier to the productive association of either unliganded and/or liganded ERα with promoter DNA, and this could underlie the previous observation that BRCA1 can inhibit ligand-dependent ERα transactivation (17, 18).

Interestingly, we observed that a deficiency of BRCA1 also leads to a reduction in the relative level of E2-mediated ERα activation. In both Brca1-nullizygous MEFs and BRCA1-deficient BG-1 (AS4) cells, the relative level of E2-mediated activation of a transfected ERα-responsive reporter gene was diminished when compared with Brca1-proficient cells. Furthermore, in AS4 cells, the endogenous estrogen-response genes that we monitored exhibited increased estrogen-independent expression and little or no estrogen-dependent stimulation when compared with BRCA1-proficient BG-1 (NEO1) cells. It is possible that the expression of these genes is largely derepressed in a BRCA1-deficient background and cannot therefore be increased substantially in response to estrogen.

Previously, Annab *et al.* (22) demonstrated that relative to parental or retroviral vector-infected BG-1 cell clones, BRCA1 antisense-infected BG-1 cell clones exhibit enhanced estrogen-independent growth in culture (22). Furthermore, BG-1 clone AS4, which exhibits severely reduced BRCA1 expression levels, exhibited increased tumorigenicity in ovariectomized nude mice compared with the retroviral vector-infected NEO1 cell clone (22). These observations suggest that forced reduction of

BRCA1 in BG-1 ovarian adenocarcinoma cells may influence estrogen-independent growth both *in vitro* and *in vivo*. Our observation that AS4 cells support significant increases in the estrogen-independent expression levels of different ERα-target genes compared with BRCA1-proficient NEO1 cells may provide a mechanistic basis for the estrogen-independent growth advantages that AS4 cells exhibit.

The finding that BRCA1 can function as a ligand-reversible barrier to transcriptional activation by unliganded ERα suggests the potential involvement of BRCA1 in the proliferative control of normal estrogen-regulated tissues. Thus, mutational inactivation of BRCA1 could result in persistent expression of estrogen-responsive genes in the absence of threshold levels of estrogenic stimulation. In this way, inappropriate hormonal responses brought about by BRCA1 mutation might possibly promote the proliferation of transformation-initiated cells.

Previous analyses have revealed that a significant proportion of BRCA1-associated breast tumors are negative for ERα expression (34). However, the loss of ERα expression in BRCA1-associated tumors is likely to represent a relatively late event in breast tumor progression, one that may have occurred after any proliferative advantages conferred upon transformation-initiated cells by homozygous BRCA1 mutation have ensued. Possibly, the down-regulation of ERα expression in BRCA1-mutated tumors could derive in part from negative feedback control enlisted by BRCA1-mutated breast epithelial cells to restrict the promiscuous expression of estrogen-responsive genes. Future studies should illuminate the mechanistic basis for BRCA1-mediated transcriptional repression of ERα and clarify its functional role in the larger network of hormone signaling pathways that control the growth, differentiation, and homeostasis of breast and ovary.

We thank D. Jones and P. Garza for technical assistance, Drs. M. J. Tsai and B. W. O'Malley for the receptor expression and reporter plasmids, Dr. J. H. White for the GAL4-VP16-ERα expression plasmid, and Dr. P.-L. Chen, Dr. P. R. Yew, and W. Tan for advice and comments. This work was supported by National Institutes of Health Grants P01CA30195 and P01CA81020, the McDermott Endowment Fund, and a San Antonio Cancer Institute Pilot Project Grant.

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0888-8809/07/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 21(7):1526–1536

doi: 10.1210/me.2007-0064

Appendix 3

Modulation of Estrogen Receptor α Protein Level and Survival Function by DBC-1

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Acquired resistance to endocrine therapy represents a major clinical obstacle to the successful management of estrogen-dependent breast cancers expressing estrogen receptor α (ERα). Because a switch from ligand-dependent to ligand-independent activation of ERα-regulated breast cancer cell growth and survival may define a path to endocrine resistance, enhanced mechanistic insight concerning the ligand-independent fate and function of ERα, including a more complete inventory of its ligand-independent cofactors, could identify novel markers of endocrine resistance and possible targets for therapeutic intervention in breast cancer. Here, we identify the deleted in breast cancer 1 gene product DBC-1 (KIAA1967) to be a principal determinant of unliganded ERα expression and survival function in human breast cancer cells. The DBC-1 amino terminus binds directly to the ERα hormone-binding domain both *in vitro* and *in vivo* in a strict ligand-independent manner. Furthermore, like estrogen, the antiestrogens tamoxifen and ICI 182,780 (7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) disrupt the DBC-1/ERα interaction, thus revealing the DBC-1/ERα interface to be a heretofore-unrecognized target of endocrine compounds commonly used in hormonal therapy. Notably, RNA interference-mediated DBC-1 depletion reduces the steady-state level of unliganded but not liganded ERα protein, suggesting that DBC-1 may stabilize unliganded ERα by virtue of their direct association. Finally, DBC-1 depletion promotes hormone-independent apoptosis of ERα-positive, but not ERα-negative, breast cancer cells in a manner reversible by endocrine agents that disrupt the DBC-1/ERα interaction. Collectively, these findings establish a principal biological function for DBC-1 in the modulation of ERα expression and hormone-independent breast cancer cell survival. (*Molecular Endocrinology* 21: 1526–1536, 2007)

BREAST CANCER IS the leading cause of death among American women between the ages of 20 and 59 yr (1). Among a variety of established etiological factors linked to breast cancer, the steroid hormone estrogen [17-β-estradiol (E2)] has long been implicated in disease pathogenesis. Numerous animal studies have revealed that E2 can induce and promote breast cancer, whereas estrogen ablation therapy or the administration of antiestrogens can oppose these effects (2–4). The physiological effects of E2 in the breast are mediated by cognate receptors that are expressed as two structurally related subtypes, estrogen receptor α (ERα) and β (ERβ) (5–8). ERα is the predominant receptor isoform expressed in breast cancer cells, and approximately 70% of breast cancer patients score positive for ERα at diagnosis (9–12). ERα is therefore a dominant etiologic and valuable predictive factor with respect to breast cancer development and hormone sensitivity status. Endocrine therapy, which seeks to block ER-mediated mitogenic signaling, has emerged as one of the most important systemic therapies in breast cancer management; however, therapeutic resistance, either inherent (*de novo* resistance) or acquired during treatment (acquired resistance) remains a significant clinical roadblock to effective disease management (13).

Although *de novo* resistance to endocrine therapy derives primarily from loss of ERα expression, the biological mechanism underlying acquired endocrine resistance is incompletely understood and almost certainly multifactorial in nature (14, 15). Nonetheless, the emergence of endocrine resistance is often coincident with a shift from ligand-dependent to ligand-independent control of ERα-regulated breast cancer cell growth and survival, possibly reflecting bidirectional molecular crosstalk between ERα and growth factor signaling pathways (14, 16, 17). Because ligand-independent activation of ERα may therefore define a path to endocrine resistance, enhanced mechanistic insight concerning the ligand-independent function and regulation of ERα, including a more complete inventory of its ligand-independent cofactors, could identify novel prognostic markers of endocrine resistance and possible

First Published Online May 1, 2007

Abbreviations: CYP40, Cyclophilin 40; E2, 17-β-estradiol; ER, estrogen receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione *S*-transferase; HSP90, heat shock protein 90; NF-κB, nuclear factor κB; NP-40, Nonidet P-40; RNAi, RNA interference; siRNA, small interfering RNA.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

targets for therapeutic intervention in breast cancer. Toward this objective, we have undertaken a proteomics-based approach to isolate ligand-independent ERα protein interaction networks. Herein, we identify the deleted in breast cancer-1 gene product DBC-1 (KIAA1967) to be a direct ligand-independent binding partner of ERα. Functional analyses further reveal DBC-1 to be a principal determinant of unliganded ERα protein levels and survival activity in human breast cancer cells.

The gene encoding DBC-1 was originally identified during a genetic search for candidate breast tumor suppressor genes on a human chromosome 8p21 region frequently deleted in breast cancers. However, refined deletion analysis within this region revealed a second gene, deleted in breast cancer 2 (*DBC-2*), to encode a likely breast tumor suppressor, and further confirmed that *DBC-1* expression is not substantially extinguished in cancers from any source (18). In fact, a search of the Oncomine database of published cancer microarray data (www.oncomine.org), which currently permits analysis of gene expression data derived from 132 DNA microarray datasets among 24 different cancer types, reveals DBC-1 to be statistically significantly upregulated in breast carcinoma *vs.* normal breast tissue as well as breast ductal carcinoma *vs.* other cancers (19, 20). Furthermore, DBC-1 was found in three independent studies totaling 369 breast tumor samples to be statistically significantly overexpressed in ER-positive *vs.* ER-negative breast tumors (21–23).

Little is currently known regarding the molecular and cellular function of DBC-1 in breast or other tissues. Recently, DBC-1 was linked physically to the TNF-α/nuclear factor κB (NF-κB) pathway by proteomic analysis (24), whereas caspase-dependent processing of DBC-1 early in apoptosis induced by diverse stimuli, including TNF-α, was shown to unmask a proapoptotic function for the DBC-1 carboxyl terminus in the cytosol of moribund cells (25). However, full-length DBC-1 is predominantly localized to the nucleus of healthy cells (25), and its normal biological function therein has heretofore remained unknown. Based on our identification of DBC-1 as a ligand-independent ERα-interacting protein as well as its provocative expression profile in breast cancers, we therefore undertook to explore the physical basis, biological regulation, and functional consequence of the interaction between DBC-1 and ERα in human breast cancer cells. Our findings reveal that the DBC-1 amino terminus binds directly to the ERα hormone-binding domain both *in vitro* and *in vivo* in a strict E2-independent manner. Furthermore, like E2, the antiestrogens tamoxifen and ICI 182,780 (7α,17β-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) disrupt the DBC-1/ERα interaction, thus revealing the DBC-1/ERα interface to be an unanticipated target of these endocrine compounds. Finally, DBC-1, in a manner dependent on direct interaction with ERα, suppresses breast cancer cell apoptosis in the absence of hormone. These findings thus establish a principal biological function for DBC-1 in the modulation of ERα expression and survival activity and further identify DBC-1 as a possible endocrine response determinant and potential therapeutic target in breast cancer.

RESULTS

DBC-1 Interacts with ERα *in Vivo* in a Ligand-Independent Manner

During the course of a targeted search for ligand-independent ERα interaction partners, we identified DBC-1 by mass spectrometric-based peptide sequence analysis of proteins coimmunoprecipitated specifically with unliganded, but not liganded, ERα (supplemental Fig. 1, which is published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). To validate the ligand-independent interaction between DBC-1 and ERα *in vivo*, we used a mammalian two-hybrid interaction analysis. Chimeric proteins consisting of DBC-1 fused to the GAL4 DNA-binding domain and ERα fused to the VP16 activation domain were expressed with or without one another in HeLa cells and examined for their respective abilities to activate transcription from a reporter template controlled by GAL4 DNA-binding sites in both the absence and presence of E2. In the absence of E2, DBC-1 and ERα exhibited a robust interaction that was disrupted by addition of E2 to the cell culture medium (Fig. 1A). Additional analysis of DBC-1 amino and carboxyl truncation derivatives revealed that the ligand-independent association between DBC-1 and ERα is mediated entirely by the amino-terminal half of DBC-1 (Fig. 1B).

To confirm the ligand-independent *in vivo* association between DBC-1 and ERα using a more biologically relevant approach, we examined the ability of antibodies specific for ERα or DBC-1 to coprecipitate one another in MCF-7 human breast cancer cells, which express both ERα and DBC-1. This analysis revealed that DBC-1 was specifically and reciprocally coimmunoprecipitated along with unliganded, but not liganded, ERα, demonstrating that the two endogenous proteins interact in a strict ligand-independent manner in human breast cancer cells (Fig. 1C). We also confirmed a ligand-independent interaction between endogenous DBC-1 and ERα in both T-47D human breast and BG-1 human ovarian cancer cell lines, thus revealing the DBC-1/ERα interaction to be conserved in a variety of ERα-expressing cell lines (Fig. 1D).

Heat shock protein 90 (HSP90) together with additional heat shock family members and immunophilins are known to form a heteromeric chaperone complex that sequesters neosynthesized and unliganded ERα in an inactive state, primes it for ligand binding, and protects it from proteolytic degradation (26–28). We initially examined the physical relationship between unliganded ERα in complex with HSP90-based chap-

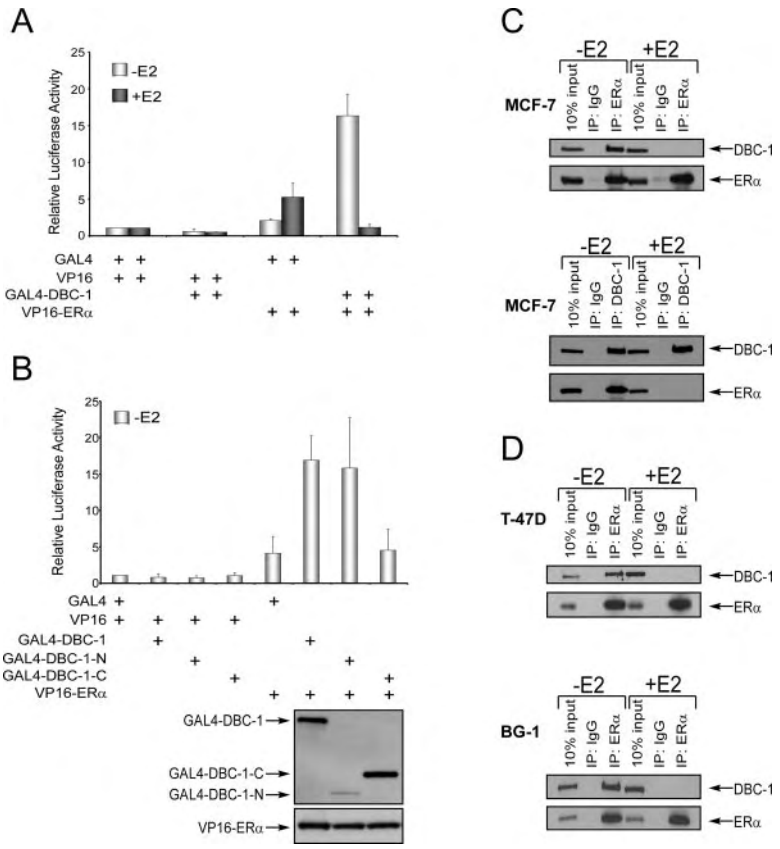

Fig. 1. DBC-1 and ERα Interact *In Vivo* in a Ligand-Independent Manner

A, B, Mammalian two-hybrid interaction analysis. A, HeLa cells cultured in hormone-free medium for 3 d were transfected with the indicated combinations of mammalian expression plasmids encoding the yeast GAL4 DNA-binding domain (GAL4), the *Herpes simplex* virus VP16 transactivation domain (VP16), a GAL4-DBC-1 chimera, and a VP16-ERα chimera. Twenty-four hours after transfection, cells were treated without (−E2) or with (+E2) E2 (10^{-7} M) for an additional 24 h before cell harvest and assay of transfected whole-cell lysates for luciferase activity produced from a cotransfected GAL4 DNA-binding site driven-reporter template. Luciferase values are expressed relative to the luciferase activity obtained in cells transfected with both the GAL4 and VP16 expression vectors, which was arbitrarily assigned a value of 1. Luciferase activities were first normalized to β-galactosidase activity obtained by cotransfection of a β-galactosidase expression vector. *Error bars* represent the SD from the average of at least three independent transfections performed in duplicate. Note that estrogen abolishes the interaction between GAL4-DBC-1 and VP16-ERα. B, *Top*, HeLa cells cultured for 3 d in hormone-free medium (−E2) were transfected with the indicated combinations of mammalian expression plasmids encoding GAL4, VP16, a GAL4-DBC-1 N-terminal chimera (amino acids 1–478), a GAL4-DBC-1 C-terminal chimera (amino acids 479–923), and a VP16-ERα chimera. Forty-eight hours after transfection, cells were harvested, and transfected whole-cell lysates were assayed for luciferase activity produced from a cotransfected GAL4 DNA-binding site driven-reporter template as described in A. Note that ERα interacts exclusively with the N terminus of DBC-1. *Bottom*, Harvested whole-cell lysates were resolved by SDS-12% PAGE and processed by immunoblot analysis with antibodies specific for GAL4-DBD or ERα as indicated by *arrows*. Note that differences in the relative expression levels of the GAL4-DBC-1 chimeras cannot explain differences in their respective ERα-binding capabilities. Results are representative of at least three independent experiments. C, D, Coimmunoprecipitation analysis. C, MCF-7 cells cultured in hormone-free medium for 3 d were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h before cell harvest and immunoprecipitation (IP) of whole-cell lysates with antibodies specific for ERα (*top*) or DBC-1 (*bottom*). Immunoprecipitates were resolved by SDS-10% PAGE and processed by immunoblot analysis using antibodies specific for DBC-1 or ERα as indicated by *arrows*. Note specific immunoprecipitation of DBC-1 by ERα-specific antibodies and ERα by DBC-1-specific antibodies only in the absence, but not in the presence, of estrogen. Results are representative of at least three independent experiments. D, T-47D (*top*) and BG-1 (*bottom*) cells cultured in hormone-free medium for 3 d were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h before cell harvest and immunoprecipitation of whole-cell lysates with antibodies specific for ERα. Immunoprecipitates were resolved by SDS-10% PAGE and processed by immunoblot analysis using antibodies specific for DBC-1 or ERα as indicated by *arrows*. Results are representative of at least three independent experiments.

erones and DBC-1 by coimmunoprecipitation analysis using MCF-7 whole-cell lysates. Whereas unliganded ERα immunoprecipitates included not only DBC-1 but also HSP90 (data not shown), DBC-1 immunoprecipitates included unliganded ERα but neither HSP90 nor the immunophilin cyclophilin 40 (CYP40) (Fig. 2A). Thus, DBC-1 is not a component of the classical HSP90-based molecular chaperone complex. Subse-

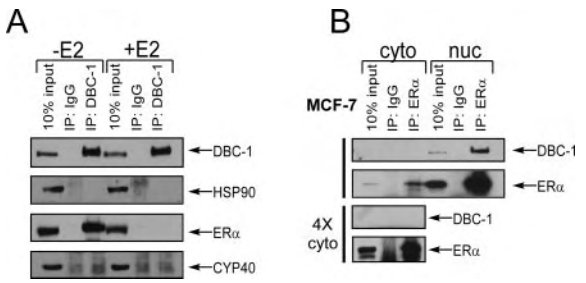

Fig. 2. DBC-1 and Unliganded ERα Associate in the Nucleus Independently of HSP90

A and B, Coimmunoprecipitation analysis. A, MCF-7 cells cultured in hormone-free medium for 3 d were treated without (−E2) or with (+E2) E2 (10^{-7} M) for 1 h before cell harvest and immunoprecipitation (IP) of whole-cell lysates with antibodies specific for DBC-1. Immunoprecipitates were resolved by SDS-10% PAGE and processed for immunoblot analysis with antibodies specific for DBC-1, HSP90, ERα, or CYP40 as indicated by *arrows*. Results are representative of at least three independent experiments. B, MCF-7 cells cultured in hormone-free medium for 3 d were fractionated into cytoplasmic (cyto) and nuclear (nuc) extracts. Equivalent amounts of each extract were immunoprecipitated with antibodies specific for ERα. Immunoprecipitates were resolved by SDS-10% PAGE and processed for immunoblot analysis with antibodies specific for DBC-1 or ERα as indicated by *arrows*. Note that an additional immunoprecipitation containing four times the amount of cytoplasmic extract (4× cyto) failed to yield a detectable amount of DBC-1 in either the input or immunoprecipitate. Results are representative of at least three independent experiments.

quently, we sought to identify the subcellular pool of unliganded ERα in specific association with DBC-1 by coimmunoprecipitation analysis using fractionated MCF-7 cell lysates. ERα/DBC-1 complexes were found exclusively in the nuclear fraction (Fig. 2B), thus revealing that unliganded ERα is distributed among at least two distinct protein complexes in human breast cancer cells: a cytosolic HSP90-based molecular chaperone complex and a nuclear DBC-1-containing protein complex.

The DBC-1 N Terminus Interacts Directly with the ERα Hormone-Binding Domain *In Vitro*

To determine whether DBC-1 interacts directly with unliganded ERα and to map the reciprocal binding domains on each protein, we tested the ability of glutathione *S*-transferase (GST)-ERα derivatives to bind to full-length DBC-1 or DBC-1 truncation fragments produced by *in vitro* translation. DBC-1 bound most efficiently to GST-ERα derivatives 1–595 (full-length ERα) and 302–595 (ERα hormone-binding domain), although DBC-1 also exhibited weak binding to GST-ERα derivative 251–301 (ERα hinge region) (Fig. 3A). Reciprocally, GST-ERα 1–595 (full-length ERα) bound to the extreme amino terminus of DBC-1 (amino acids 1–150) (Fig. 3B). Thus, in the absence of ligand, the ERα hormone-binding domain can accommodate the DBC-1 amino terminus.

The DBC-1/ERα Interface Is a Novel Target of Antiestrogens

Antiestrogens are currently the most widely administered endocrine agents for the management of ERα-expressing breast cancers (29, 30). Mechanistically, antiestrogens competitively displace E2 from the ERα hormone-binding domain and either block ERα function or induce destabilization and degradation of ERα. Tamoxifen, a prototype of the former class, is a selective ER modulator with antiestrogenic properties in breast and the most widely administered antiestrogen in breast cancer therapy (29, 30). Among the latter class of antiestrogens, ICI 182,780 (Faslodex; fulvestrant) is a selective ER down-regulator and an effective second-line therapeutic agent used to treat breast cancers that have progressed on previous tamoxifen therapy (29–31). Because these compounds bind directly to the ERα hormone-binding domain, we examined the influence of each agent on the DBC-1/ERα interaction. To this end, we tested the ability of ERα-specific antibodies to coimmunoprecipitate endogenous DBC-1 present in MCF-7 and BG-1 cells cultured in the absence or presence of E2, tamoxifen, or ICI 182,780. Strikingly, we observed that, like E2, both tamoxifen and ICI 182,780 disrupted the DBC-1/ERα interaction, thus revealing the DBC-1/ERα interface to be a heretofore unrecognized target of these endocrine compounds (Fig. 4, A and B).

DBC-1 Is an ERα-Dependent Prosurvival Factor in Breast Cancer Cells

To examine the biological consequence of the DBC-1/ERα interaction in human breast cancer cells, we first established conditions for RNA interference (RNAi)-mediated DBC-1 depletion in MCF-7 cells. Strikingly, we observed that RNAi-mediated DBC-1 knockdown was accompanied by a significant reduction in the steady-state level of ERα protein but not ERα mRNA, suggesting that DBC-1 modulates ERα protein synthesis or stability (Fig. 5). Notably, DBC-1 knockdown preferentially reduced the steady-state level of unliganded, but not liganded, ERα protein, consistent with the possibility that DBC-1 may stabilize unliganded ERα by virtue of their direct physical association (Fig. 5).

Because DBC-1 is a direct binding partner and key determinant of steady-state ERα protein levels, we examined its role in ERα-dependent breast cancer cell proliferation and survival. RNAi-mediated DBC-1 depletion significantly reduced E2-independent, but not E2-dependent, MCF-7 cell proliferation, an observation concordant with the fact that DBC-1 preferentially binds to and modulates the levels of unliganded ERα (Fig. 6). Because transient DBC-1 knockdown cells experienced an initial (~2-fold) reduction in cell number on d 3 after small interfering RNA (siRNA) delivery followed by growth kinetics similar to control siRNA knockdown cells, we hypothesized that the influence

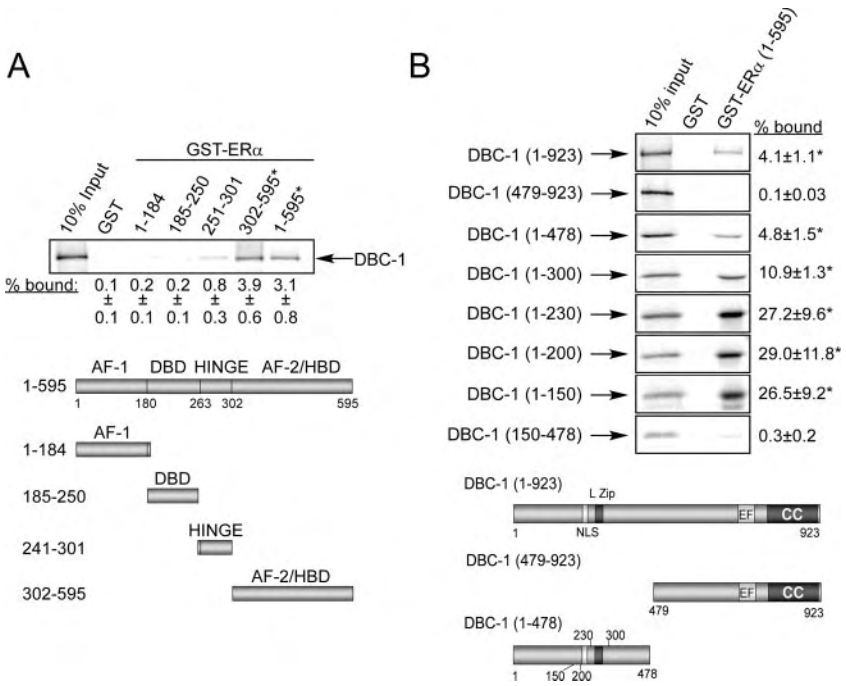

Fig. 3. The DBC-1 N Terminus Binds to the ERα Hormone-Binding Domain *in Vitro*

A and B, GST pull-down assays were performed using full-length *in vitro* translated DBC-1 and GST-ERα fragments (A) or *in vitro* translated DBC-1 fragments and GST-full-length ERα (B) as indicated. Numbers refer to amino acid coordinates. ^{35}S-labeled *in vitro* translated proteins were incubated with glutathione-Sepharose-immobilized GST derivatives, and bound proteins were resolved by SDS-12% PAGE before detection by PhosphorImager analysis. Input represents 10% of the ^{35}S-labeled *in vitro* translated proteins used in binding reactions. The amount of each DBC-1 derivative retained by GST-ERα (percentage bound) was quantified and expressed as a percentage of the total input. % bound refers to the average and SD of at least three independent experiments. *, $P < 0.05$, statistically significant binding values relative to GST alone. Note that DBC-1 binds primarily to GST-ERα derivatives 1–595 (full-length ERα) and 302–595 (ERα hormone-binding domain), whereas GST-ERα binds primarily to DBC-1 derivative 1–150 (N terminus). Schematic diagrams of ERα and DBC-1 indicate fragments used in binding reactions. AF-1, Activation function 1; DBD, DNA-binding domain; AF-2/HBD, activation function 2/hormone-binding domain; NLS, putative nuclear localization sequence; LZip, putative leucine zipper.

of DBC-1 silencing on ligand-independent cell proliferation may derive, at least in part, from an increase in apoptotic cell death. To address this question, we examined the influence of DBC-1 knockdown on the apoptotic fate of MCF-7 cells cultured in the absence of E2. Under these conditions, DBC-1 depletion increased the percentage of apoptotic cells from 6.2 to 12.8%, thus revealing an antiapoptotic function for DBC-1 in the absence of hormone (Fig. 7A). To determine whether DBC-1 promotes hormone-independent cell survival through its direct interaction with ERα, we also monitored the influence of DBC-1 knockdown on the apoptotic fate of MCF-7 cells cultured in the presence of E2, which disrupts the DBC-1/ERα interaction, or ICI 182,780, which not only disrupts the DBC-1/ERα interaction but also drastically depletes ERα protein levels. Notably, DBC-1 depletion had no effect on MCF-7 cell apoptosis in the presence of either E2 or ICI 182,780 (Fig. 7A). Furthermore, DBC-1 depletion did not enhance apoptosis of ERα-negative MDA-MB-231 breast cancer cells cultured in the absence of E2 (Fig. 7B). Together, these observations suggest that DBC-1 functions to promote E2-independent breast cancer cell survival in an ERα-dependent manner.

We note that we have also attempted to examine the influence of DBC-1 overexpression on breast cancer cell proliferation and survival; however, we have not been able to achieve overexpression of DBC-1 protein in ERα-expressing breast cancer cells, suggesting that DBC-1 expression levels are tightly regulated in this context.

DISCUSSION

Here we describe for the first time a biological function for DBC-1 in the modulation of ERα expression and survival activity in human breast cancer cells. Our identification of DBC-1 as a heretofore unrecognized determinant of steady-state ERα protein levels is a novel finding with implications for the regulation and function of ERα in normal and malignant breast epithelial cells. A compelling body of experimental, clinical, and epidemiological evidence suggests that dysregulation of ERα expression is a driving force in the initiation and progression of estrogen-sensitive breast tumors. ERα is the predominant receptor isoform ex-

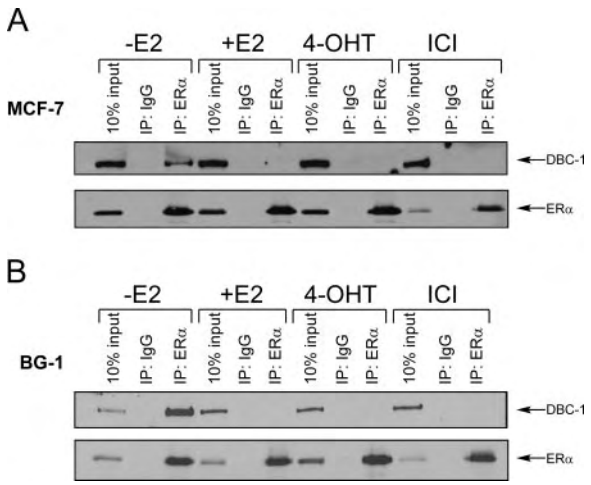

Fig. 4. Tamoxifen and ICI 182,780 Disrupt the Interaction between DBC-1 and ERα

A and B, MCF-7 (A) or BG-1 (B) cells cultured in hormone-free medium for 3 d were treated with vehicle (−E2), E2 (10^{-7} M; +E2), 4-hydroxytamoxifen (10^{-6} M; 4-OHT), or ICI 182,780 (10^{-7} M; ICI) for 1 h before cell harvest and immunoprecipitation (IP) of whole-cell lysates with antibodies specific for ERα. Immunoprecipitates were resolved by SDS-7.5% PAGE and processed for immunoblot analysis with antibodies specific for DBC-1 or ERα as indicated by *arrows*. Results are representative of at least three independent experiments.

pressed in breast cancer cells, and increased numbers of ERα-expressing cells as well as increased individual cell ERα content can be observed at the earliest stages of breast tumorigenesis (32, 33). It is thus likely that alterations in pathways leading to ERα synthesis and/or degradation underlie the dysregulation of ERα and its consequent manifestations, including enhanced proliferation in breast tumors. Therefore, revelation of the mechanism by which DBC-1 modulates ERα expression should yield important insight concerning the physiological regulation and, possibly, the pathological dysregulation of ERα in normal and malignant breast epithelial cells, respectively.

In this regard, previous work has revealed that unliganded ERα is sequestered by an HSP90-based molecular chaperone complex that protects the neosynthesized receptor from proteolytic degradation and primes it for ligand binding (26, 27, 34, 35). Our observation that HSP90 and CYP40 cannot be coprecipitated along with unliganded ERα by DBC-1-specific antibodies coupled with our finding that DBC-1 and unliganded ERα interact in the nucleus suggests that the cellular reserve of unliganded ERα is partitioned among at least two pools: one comprising cytosolic HSP90-based molecular chaperones and the other nuclear DBC-1. Notably, we observed that DBC-1 depletion preferentially reduced the steady-state level of unliganded ERα protein, suggesting the possibility that unliganded ERα is stabilized by its direct physical association with DBC-1. Thus, DBC-1 could function as a chaperone of ERα in the nucleus in a manner analogous to that of HSP90 toward ERα in the cytosol. Additional studies will be required to elucidate the mechanism by which DBC-1 modulates ERα steady-state protein levels.

Several observations herein suggest a novel anti-apoptotic function for the population of unliganded ERα bound by DBC-1. First, apoptosis triggered by DBC-1 depletion in the absence of hormone was not observed in MCF-7 cells codepleted of ERα with ICI 182,780, nor in ERα-negative MDA-MB-231 breast cancer cells. These findings thus reveal an apparent DBC-1-dependent ERα requirement for suppression of apoptosis in the absence of hormone. Second, E2-mediated disruption of the interaction between DBC-1 and unliganded ERα abrogated the increase in MCF-7 cell apoptosis observed to accompany DBC-1 knockdown, suggesting that DBC-1-bound ERα functions to suppress hormone-independent apoptosis. We therefore speculate that a specific pool of unliganded ERα bound by DBC-1 may promote breast cancer cell growth and survival in the absence of hormone.

The underlying mechanism by which DBC-1 and ERα collaborate to promote hormone-independent breast cancer cell growth and survival remains to be established. As discussed above, DBC-1 could directly stabilize a pool of unliganded ERα dedicated to these functions. Whether or not DBC-1 additionally directly participates in ERα-regulated cell growth and survival processes is presently unknown. An intriguing possibility is that DBC-1 might function to mediate crosstalk between ERα and the NF-κB survival pathway. Emerging evidence indicates that bidirectional molecular crosstalk between the ERα and NF-κB pathway contributes to hormone-independent breast cancer cell growth and the development of antiestrogen resistance (36–40). Previously, DBC-1 has been linked physically to the NF-κB pathway through a demonstrated interaction with IκB kinase β (24), although our findings herein link DBC-1 physically and functionally to ERα. Possibly, DBC-1 could thus serve to stabilize and channel ERα toward functional interactions with the NF-κB pathway. Future studies will be required to establish whether and how DBC-1-mediated crosstalk between the ERα and NF-κB signaling pathways might contribute to hormone-independent breast cancer cell growth and survival.

Finally, our finding that ERα and DBC-1 collaborate to suppress apoptosis and promote hormone-independent breast cancer cell growth could have implications for breast cancer prognosis and/or treatment. It is generally believed that a balance between proliferation and apoptosis influences the response of breast tumors to hormonal therapy, and dysregulation of apoptotic signaling pathways has been suggested as a possible basis for treatment failure (29, 30, 41, 42). Accordingly, alterations in DBC-1 expression and/or activity could tip the balance between breast cancer cell growth and death; if so, DBC-1 could represent a novel biomarker of breast tumor response to endocrine therapy. In this regard, no published data

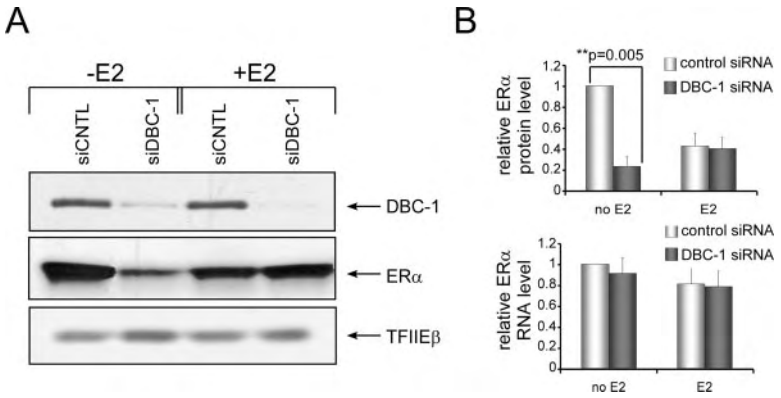

Fig. 5. RNAi-Mediated DBC-1 Suppression Is Accompanied by Reduced Steady-State Levels of Unliganded ERα

MCF-7 cells cultured in hormone-free medium for 3 d were electroporated with control (siCNTL) or DBC-1-specific (siDBC-1) siRNA (21 nM) as indicated. Electroporated cells were cultured without (−E2) or with (+E2) E2 (10^{-7} M) for an additional 3 d before cell harvest. A, Harvested whole-cell lysates were resolved by SDS-10% PAGE and processed by immunoblot analysis with antibodies specific for DBC-1, ERα, or TFIIEβ as indicated by *arrows*. Results are representative of at least three independent experiments. B, *Top*, Immune signals were quantified using a Kodak ImageStation 2000R. ERα protein levels were normalized to TFIIEβ and plotted relative to the ERα protein level in control siRNA cells cultured in the absence of E2, which was arbitrarily assigned a value of 1. *Error bars* represent the SD from the average of at least three independent experiments. *Bottom*, RNA was processed by quantitative RT-PCR analysis for the levels of DBC-1, ERα, and GAPDH mRNAs. ERα RNA levels were normalized to GAPDH levels and expressed relative to the level of ERα RNA in control siRNA cells cultured in the absence of E2, which was arbitrarily assigned a value of 1. *Error bars* represent the SD from the average of at least three independent experiments performed in duplicate.

currently exists concerning the relationship between DBC-1 and clinical response of breast tumors to endocrine therapy. Nonetheless, it would be useful to know whether overexpression or amplification of DBC-1 is linked to treatment failure. Furthermore, although DBC-1 is not deleted in most breast cancers, it would be of interest to know the hormone receptor and endocrine response status of the relatively small percentage of breast cancers that do harbor DBC-1 deletions. For example, might ER-positive patients carrying DBC-1 deletions be underrepresented among the patient pool refractory to endocrine therapy? Answers to these and related questions should help to clarify the possible role of DBC-1 as a predictor of breast tumor response to endocrine therapy. From a possible therapeutic perspective, disruption of the DBC-1/ERα interface might provide a targeted means to reduce in breast tumors the number of hormone-refractory cells that arise through selection in response to prolonged endocrine treatment. Future experiments will be required to validate this hypothesis and further investigate the full spectrum of ERα-dependent and ERα-independent biological activities linked to DBC-1.

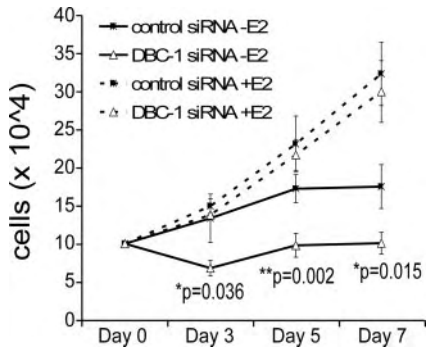

Fig. 6. RNAi-Mediated DBC-1 Depletion Inhibits Estrogen-Independent Proliferation in Human Breast Cancer Cells

MCF-7 cells cultured in hormone-free medium for 3 d were electroporated with control or DBC-1-specific siRNA (21 nM) as indicated and cultured without (−E2) or with (+E2) E2 (10^{-7} M). Culture medium was replaced every 2 d. Cell proliferation was monitored by counting with trypan blue exclusion for 7 d after electroporation. *P* values are compared with controls. *Error bars* represent the SD from the average of at least three independent experiments performed in triplicate.

MATERIALS AND METHODS

Expression Plasmids

pCS2+-ERα was constructed by subcloning a *Bam*HI-*Bam*HI fragment carrying the full-length coding region of ERα cDNA from pG/ER(G) (provided by Dr. Dider Picard, University of Geneva, Geneva, Switzerland) (43) into pCS2+ (44). pACT-ERα was constructed by subcloning a *Bam*HI-*Bam*HI fragment carrying the full-length coding region of ERα cDNA from pCS2+-ERα into the pACT VP16 fusion vector (Promega, Madison, WI). GST-ERα (1–184), GST-ERα (185–250), GST-ERα (251–301), and GST-ERα (302–595) were gifts from Dr. Yi-Jun Zhu (Northwestern University, Evanston, IL) (45). GST-ERα (1–595) was generated by amplifying ERα by PCR and inserting it into the *Eco*RI site of pGEX-4T-3 vector (GE Healthcare, Little Chalfont, UK).

pSport1-DBC-1 was a clone obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung (www.rzpd.de) (RZPD clone DKFZp761O0817Q; KIAA1967). pCS2+DBC-1 was constructed by first amplifying the amino-terminal half of

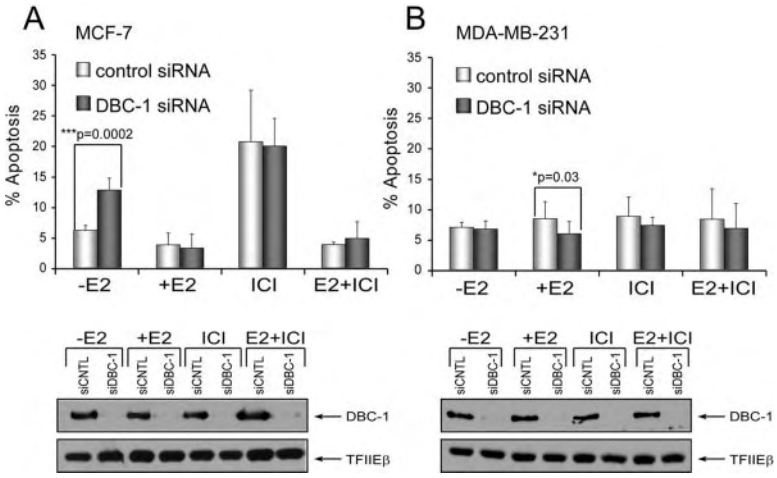

Fig. 7. DBC-1 Is an ERα-Dependent Prosurvival Factor in Human Breast Cancer Cells

A and B, MCF-7 (A) or MDA-MB-231 (B) cells cultured in hormone-free medium for 3 d were electroporated with control or DBC-1-specific siRNA (21 nM) as indicated. Forty-eight hours after electroporation, cells were treated with vehicle (−E2), E2 (10^{-7} M; +E2), ICI 182,780 (10^{-7} M; ICI), or a combination of E2 and ICI 182,780 (E2+ICI) for an additional 24 h before cell harvest. *Top*, Harvested cells were stained with Annexin V-FITC and propidium iodide before quantification of apoptosis by flow cytometric analyses. *P* values are compared with controls. *Error bars* represent the SD from the average of at least three independent experiments performed in triplicate. *Bottom*, Cell lysates from representative apoptosis assays in A and B were resolved by SDS-10% PAGE and processed by immunoblot analysis with the indicated antibodies specific for DBC-1 or TFIIEβ as a loading control.

DBC-1 by PCR and inserting it into the *Cla*I/*Eco*RI site of pCS2+.His6.FLAG, which yielded pCS2+.His6.FLAG-5′DBC-1.SphI. The carboxyl-terminal half of DBC-1 was amplified by PCR and then inserted into the *Sph*I/*Eco*RI site of pCS2+.His6.FLAG-5′DBC-1.SphI to yield pCS2+DBC-1, which contains a STOP codon between the DBC-1 coding sequence and the His6.FLAG fusion. This construct was confirmed by sequencing. pCS2+.His6.FLAG-DBC-1 was generated by amplifying the carboxyl-terminal half of DBC-1 by PCR and then inserting it into the *Sph*I/*Eco*RI site of pCS2+.His6.FLAG-5′DBC-1.SphI to create a version of DBC-1 fused to C-terminal 6XHis and FLAG tags. pCS2+-DBC-1 amino-terminal fragments (1–478, 1–300, 1–230, 1–200, 1–150, and 150–478) were generated by amplifying fragments by PCR and inserting them into the *Eco*RI/*Xho*I site of pCS2+. pCS2+-DBC-1 (479–923) was generated by amplifying the carboxyl-terminal half of DBC-1 by PCR and inserting it into the *Eco*RI/*Xho*I site of pCS2+. pBIND-DBC1 (1–478) was constructed by amplifying the amino-terminal half of DBC-1 by PCR and inserting it into the *Sal*I/*Xba*I site of the pBIND GAL4 fusion vector (Promega). pBIND-DBC1 (479–923) was constructed by amplifying the carboxyl-terminal half of DBC-1 by PCR and inserting it into the *Xba*I/*Not*I site of pBIND. pBIND-DBC-1 was constructed by subcloning an *Xba*I/*Not*I carboxyl-terminal fragment of DBC-1 from pBIND-DBC1 (479–923) into pBIND-DBC1 (1–478).

Reporter Plasmids

pG5luc, carrying five GAL4 DNA-binding sites upstream of the major late promoter of adenovirus driving expression of the firefly luciferase gene, was purchased from Promega.

Cell Lines and Culture Conditions

The HeLa (American Type Culture Collection, Manassas, VA), T-47D (American Type Culture Collection), MCF-7 (American Type Culture Collection), AmphoPack 293 (Clontech, Mountain View, CA), and MDA-MB-231 (American Type Culture Collection) cells were routinely cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and penicillin-streptomycin-L-glutamine (Invitrogen). BG-1 cells, from Dr. Kenneth S. Korach (National Institute of Environmental Health Sciences, Research Triangle Park, NC) (46), were routinely cultured in DMEM/F12 (Invitrogen) supplemented as listed above. All cell lines except BG-1 and MDA-MB-231 cells were cultured at 37 C in a 10% CO_2 humidified chamber; BG-1 and MDA-MB-231 cells were cultured at 5% CO_2.

GST Pull-Down Assays

GST and GST fusion proteins were expressed in and purified from BL21-CodonPlus(DE3)-RIPL *Escherichia coli* (Stratagene, La Jolla, CA). Cells were grown at 37 C to A_{600} of 1.0, and then isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. For GST, GST-ERα (1–184), GST-ERα (185–250), and GST-ERα (251–301), the cells were grown at 30 C for another 5 h. For GST-ERα (302–595), the cells were grown at 20 C for another 5 h. For GST-ERα (1–595), the cells were grown at 16 C for another 5 h. Cells were pelleted, washed once with PBS, and resuspended in lysis 250 buffer [50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40 (NP-40)] supplemented with protease inhibitors (20 μM antipain, 2 μM pepstatin, 20 μM leupeptin, and 2 μg/ml aprotinin). Resuspended cells were subjected to one round of freeze-thaw, followed by sonication and clarification by centrifugation at 35,000 × *g* for 30 min at 4 C.

Clarified GST lysates were bound to glutathione-Sepharose beads (GE Healthcare) for 45 min at 25 C, followed by washing four times for 5 min each with lysis 250 buffer containing 0.2% BSA and protease inhibitors. DBC-1 or fragments of DBC-1 were labeled with [^{35}S]methionine (TNT SP6 quick-coupled transcription/translation system; Promega) and incubated with immobilized GST proteins in PD buffer (50 mM Tris-HCl, 200 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, and 0.05% NP-40) for 2 h at 4 C. Binding reactions were washed with PD buffer three times for 5 min each at 4 C and subsequently boiled in 20 μl of 1× Laemmli's sample buffer. Elu-

ates were resolved by SDS-12% PAGE and visualized by PhosphorImager analysis (GE Healthcare).

Mammalian Two-Hybrid Interaction Analysis

HeLa cells grown under hormone-free conditions for 2 d were plated at 1×10^5 cells per well in 12-well plates (Dow Corning, Corning, NY). After 24 h, the cells were transfected using FuGENE 6 (Roche, Indianapolis, IN) according to the recommendations of the manufacturer. In defining the ERα-DBC-1 interaction, transfection mixtures consisted of pCH110 (47), an internal control plasmid, expressing β-galactosidase under control of the simian virus 40 promoter (167 ng), pG5*luc* reporter (167 ng), pACT-ERα (334 ng), and the various pBIND-DBC-1 constructs (334 ng), including pBIND-DBC-1, pBIND-DBC-1 (1–478), and pBIND-DBC-1 (479–923). pBIND empty vector was used as an appropriate control for interaction with pACT-ERα. pACT empty vector was used as an appropriate control for interaction with the various pBIND-DBC-1 constructs. After 48 h, cells were harvested and assayed for luciferase activity according to the guidelines of the manufacturer (Promega). Luciferase activity was corrected for the corresponding β-galactosidase activity to give relative activity. β-Galactosidase activity was assayed according to the instructions of the manufacturer (Tropix, Bedford, MA). Transfections were repeated a minimum of three times in duplicate. For experiments with ligand treatment, E2 (Sigma, St. Louis, MO) was added to cells at 10^{-7} M for 24 h before harvest.

For Western blot analysis, 48 h after transfection, whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, and 0.1% SDS) supplemented with protease inhibitors and clarified by centrifugation. Equivalent amounts of lysates were boiled in Laemmli's sample buffer and resolved by SDS-10% PAGE. Proteins were analyzed by immunoblot using antibodies against GAL4-DBD (RK5C1; Santa Cruz Biotechnology, Santa Cruz, CA) and ERα (HC-20; Santa Cruz Biotechnology).

Coimmunoprecipitations

T-47D, MCF-7, or BG-1 cells were grown under hormone-free conditions for 3 d and treated without or with E2 (10^{-7} M), 4-hydroxytamoxifen (10^{-6} M; Sigma), or ICI 182,780 (10^{-7} M; Tocris, Ellisville, MO) for 1 h before cell harvest and coimmunoprecipitation. Whole-cell lysates were prepared in 0.5% NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) supplemented with protease inhibitors and clarified by centrifugation. Nuclear and cytoplasmic extracts were prepared as described previously (48). Lysates were adjusted to binding buffer (50 mM Tris-HCl, 175 mM NaCl, 5 mM EDTA, 0.2% NP-40, and 10% glycerol, supplemented with protease inhibitors) concentration. Lysates were then subjected to immunoprecipitation with rabbit polyclonal anti-ERα (HC-20; Santa Cruz Biotechnology) antibody or mouse polyclonal anti-DBC-1 antibody [produced in our laboratory against recombinant DBC-1 (amino acids 475–923)] and protein A-Sepharose beads. Immune complexes were washed three times with binding buffer, boiled in Laemmli's sample buffer, and resolved by SDS-10% PAGE. Proteins were transferred to nitrocellulose membranes and visualized by using antibodies against DBC-1, ERα, HSP90 (rabbit polyclonal; Genetex, San Antonio, TX), CYP40 (rabbit polyclonal; Abcam, Cambridge, MA), appropriate peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), and enhanced chemiluminescence detection (GE Healthcare).

DBC-1 Silencing by siRNA

To selectively knock down the expression of endogenous DBC-1 protein, an siRNA pool consisting for four different target sequences was used (catalog no. 010427; Dharmacon, Chicago, IL). These RNA duplexes (3 μg per 2×10^6 cells), as well as a negative control duplex that does not pair with any human mRNA (Dharmacon), were electroporated in MCF-7 or MDA-MB-231 cells using the cell line Nucleofector kit V (Amaxa, Gaithersburg, MD). Immediately after control or DBC-1 siRNA electroporation, cells were seeded at a concentration of 1×10^6 per 60 mm plate. In all experiments, cells were allowed to grow for 3 d in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS and without or with indicated chemical treatments. Cells were harvested 3 d after electroporation.

Western Blot Analysis

Three days after electroporation, whole-cell lysates were prepared in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, and 0.1% SDS) supplemented with protease inhibitors and clarified by centrifugation. Lysates were boiled in Laemmli's sample buffer and resolved by SDS-10% PAGE. Proteins were analyzed by immunoblot using antibodies against DBC-1 (produced in our laboratory), ERα (HC-20; Santa Cruz Biotechnology), and TFIIEβ (C-21; Santa Cruz Biotechnology) as described previously. Quantification of Western blots was performed using the Kodak ImageStation 2000R (Eastman Kodak, Rochester, NY).

Quantitative Real-Time RT-PCR

Three days after electroporation, RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA was reverse transcribed using random hexamers and Superscript III (Invitrogen) following the instructions of the manufacturer. Quantitative RT-PCR was performed using ABsolute SYBR Green ROX Mix (ABgene, Rochester, NY) on an ABI PRISM 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA). The gene-specific primers used were as follows: DBC-1, 5′-ATG TCC CAG TTT AAG CGC CAG-3′ and 5′-CAA CCC CAA AGT AGT CAT GCA A-3′; ERα, 5′-CCA CCA ACC AGT GCA CCA TT-3′ and 5′-GGT CTT TTC GTA TCC CAC CTT TC-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CCT GTT CGA CAG TCA GCC G-3′ and 5′-CGA CCA AAT CCG TTG ACT CC-3′.

Proliferation Assays

Three days before electroporation, cells were grown in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS. Immediately after control or DBC-1 siRNA electroporation, cells were seeded at a concentration of 10×10^4 per well in six-well plates. In all experiments, triplicates of cells were allowed to grow for 7 d in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS and without or with E2 (10^{-7} M) at 37 C and 10% CO_2. Cell viability was determined using the trypan blue exclusion assay, and viable cells were counted with the use of a hemacytometer. Proliferation assays were repeated a minimum of three times.

Apoptosis Assays

Three days before electroporation, cells were grown in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS. Immediately after control or DBC-1 siRNA electroporation, cells were seeded at a concentration of 1×10^6 per 60 mm plate. In all experiments, cells were allowed to grow for 3 d in phenol-red-free medium supplemented with 10% charcoal/dextran-treated FBS and without or with E2 (10^{-7} M), ICI 182,780 (10^{-7} M; Tocris), or a combination of the two at 37 C and 10% CO_2. Seventy-two hours after electro-

poration, trypsinized cells (1×10^5) were stained with Annexin V-fluorescein isothiocyanate (FITC) (BD Pharmingen, San Diego, CA) and propidium iodide (Becton Dickinson, Franklin Lakes, NJ) according to the instructions of the manufacturer. Flow-cytometric analyses to quantify apoptosis were done in an FACSCalibur (Becton Dickinson). All Annexin V-FITC-positive cells were considered apoptotic. Apoptosis assays were repeated a minimum of three times.

Data Analysis

Statistical significance was assessed by comparing mean ± SD values with Student's *t* test for independent groups. $P \leq 0.05$ was considered statistically significant.

Acknowledgments

We are grateful for the various plasmids received from D. Picard, Y. J. Zhu, and Deutsches Ressourcenzentrum für Genomforschung. We thank W. S. Lane and the Harvard Microchemistry Facility for performing the peptide sequencing by LC/MS/MS. We also thank K. Korach for providing BG-1 cells. We thank E. White for providing DBC-1 rabbit polyclonal antibody used in the initial stage of these studies. We are also grateful to P. Garza for DBC-1 antibody production. We thank P. Garza, N. Ding, W. Tan, S. Kim, H. Zhou, L. N. Ngo, P. R. Yew, R. E. Cendaña, X. N. Zhu, H. Li, V. B. Holcomb, T. Marple, and other colleagues for assistance, advice, discussion, and comments.

Received January 31, 2007. Accepted April 27, 2007.

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This work was supported by National Institutes of Health Predoctoral Fellowship 5T32CA086800-04, National Institutes of Health Grant CA098301-01, and United States Army Department of Defense Grant DAMD17-02-1-0584.

Disclosure Summary: The authors have nothing to disclose.

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Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Appendix 4

[CANCER RESEARCH 62, 3966–3970, July 15, 2002]

Deficient Nonhomologous End-Joining Activity in Cell-free Extracts from Brca1-null Fibroblasts[1]

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ABSTRACT

BRCA1 ensures genomic stability, at least in part, through a functional role in DNA damage repair. BRCA1 interacts with the Rad50/Mre11/Nbs1 complex that occupies a central role in DNA double-strand break repair mediated by homologous recombination and nonhomologous end joining (NHEJ). NHEJ can be catalyzed by mammalian whole cell extract in a reaction dependent upon DNA ligase IV, Xrcc4, Ku70, Ku80, and DNA-PKcs. Here, we show that under identical cell-free reaction conditions, the addition of antibodies specific for BRCA1 and Rad 50 but not Rad51, inhibits end-joining activity. Cell extracts derived from Brca1-deficient mouse embryonic fibroblasts exhibit reduced end-joining activity independent of the endogenous protein amounts of DNA ligase IV, Ku80, and Ku70. The Brca1-dependent NHEJ activity predominates at the lower concentrations of Mg2+ (0.5 mM); elevated Mg2+ or Mn2+ concentrations (10 mM) dramatically increase overall end-joining activity and abrogates the requirement for Brca1, Xrcc4, and Ku70. The addition of partially purified BRCA1, in association with Rad50/Mre11/Nbs1 complex, complements the NHEJ deficiency of Brca1-null fibroblast extracts. These results suggest a role for Brca1 in NHEJ and in the maintenance of genome integrity.

INTRODUCTION

Inactivation of the hereditary breast cancer susceptibility gene, *BRCA1*,[3] leads to genomic instability (1–3). Extensive chromosomal abnormalities have been observed in Brca1-deficient murine fibroblasts (4), as well as the BRCA1-mutant human breast cancer cell line HCC1937 (5). The function of BRCA1 in genome stability is attributable to its central role in the cellular response to DNA damage response, and emerging evidence supports a role for BRCA1 in DNA damage repair. For example, Brca1-deficient murine and human cells are sensitive to DNA-damaging agents, including IR (6–8). Furthermore, HCC1937 cells expressing mutant BRCA1 protein exhibit a reduction in both the rate and extent of DSB repair after IR when compared with cells expressing wild-type BRCA1 protein (9). Finally, BRCA1 physically interacts with the Rad50/Mre11/Nbs1 DSB repair complex and colocalizes to nuclear foci along with this complex after treatment of cells with IR (8).

In eukaryotic cells, DSBs are repaired through two distinct pathways: homologous recombination and NHEJ. BRCA1 has been implicated in homology-based repair because cells expressing a Brca1 exon-11 deletion mutant exhibit defects in gene targeting, single-strand annealing, and gene conversion (10). BRCA1 may also influence NHEJ by virtue of its interaction with the Rad50/Mre11/Nbs1 complex. The orthologous complex in *Saccharomyces cerevisiae* Rad50/Mre11/Xrs2 is critical for NHEJ, sister chromatid recombination, and telomere maintenance. Yeast strains deficient in any of the components of the Rad50/Mre11/Xrs2 complex are 10–100-fold less efficient in nonhomologous joining of DNA ends (11, 12). The Rad50/Mre11/Nbs1 complex is characterized by 3′ to 5′ exonucleolytic activity on double-stranded DNA and endonucleolytic activity on single-stranded DNA and hairpin structures. Furthermore, in the presence of a DNA ligase, Mre11 can facilitate DNA end joining using microhomologies at or near DNA termini (13, 14). Recently, the yeast Rad50/Mre11/Xrs2 complex was found to exhibit DNA end-binding activity and end-bridging activity (15). Thus, the Rad50/Mre11/Nbs1 complex may fulfill a functionally conserved role in NHEJ.

In mammalian cells, a NHEJ pathway has been identified that comprises the heterodimeric DNA end-binding activity Ku70/Ku80 and the DNA-PKcs (reviewed in Ref. 12). Recently, Baumann *et al.* (16) developed a cell-free system that faithfully reflects the genetic requirements for this NHEJ pathway. In this system, accurate intermolecular ligation of DNA ends was found to be dependent on DNA ligase IV/Xrcc4 and requires Ku70, Ku80, and DNA-PKcs. However, the role of Rad50/Mre11/Nbs1 in this NHEJ assay has not been addressed.

We report here the use of this cell-free assay to investigate the role of BRCA1 in DNA end joining. We observed that antibodies specific for BRCA1, Rad50, and Ku70, but not Rad51, inhibit the end-joining activity present in extracts prepared from a human lymphoblastoid cell line. Comparison of extracts derived from Brca1-null MEFs with that from isogenic Brca1-proficient MEFs for their respective abilities to catalyze end joining *in vitro* revealed that Brca1-deficient MEF extracts exhibit a significantly reduced end-joining activity. This deficiency can be complemented by partially purified BRCA1 in association with the Rad50/Mre11/Nbs1 complex. Finally, we found the BRCA1-dependent NHEJ activity in mammalian WCE to be sensitive to the reaction concentration of divalent cations. Elevated concentrations of Mg2+ or Mn2+ (to 10mM) stimulated the overall level of DNA end-joining activity and masked a BRCA1, Ku, and Xrcc4 requirement. These results provide evidence that BRCA1 promote NHEJ in a Mg2+ concentration-dependent manner.

MATERIALS AND METHODS

MEFs and Lymphoblastoid Cell Line. The *Brca1*$^{-/-}$:*p53*$^{-/-}$ and p53$^{-/-}$ MEFs were derived from 9.5-day old embryos of a cross between *Brca1*$^{+/-}$ and *p53*$^{+/-}$ mice as described (17) and cultured in DMEM plus 10% FCS. Human lymphoblastoid cell line, LEM, was immortalized by Epstein-Barr virus and cultured in DMEM plus 10% FCS.

Cell-free NHEJ Assay. Cell extracts were prepared and *in vitro* reactions were performed according to previously described procedure (16). WCEs were normalized for their respective total protein levels using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). Reactions (16 μl) were carried out in 50 mM triethanolamine-HCl (pH 7.5), 0.5 mM $Mg(OAc)_2$, 80 mM potassium acetate, 2 mM ATP, 1 mM DTT, and 100 μg/ml BSA. Cell-free extracts were incubated for 5 min at 37°C before the addition of 5 fmol ^{32}P-labeled DNA. pBSK(+) duplex plasmid DNA (2.96 kb; Stratagene, La Jolla, CA) was linearized with *Eco*RI, dephosphorylated using calf intestinal phosphatase, and was 5′ ^{32}P-end-labeled using polynucleotide kinase. In each reaction, 5 fmol of labeled DNA was used. After incubation at 37°C for 1 h, ^{32}P-labeled DNA products were deproteinized by proteinase K (500 μg/ml) and 1% SDS at 37°C for 20 min and analyzed by electrophoresis through 0.7% agarose gels, followed by autoradiography. Quantitation of DNA end-joining efficiency was carried out

Received 3/12/02; accepted 5/13/02.

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[1] Supported in part by NIH Grant CA 81020 and 94170 (to W-H. L.) and Grant CA 85605 (to P-L. C.). Q. Z. was supported by a predoctoral training grant from the U.S. Army Medical Research and Materiel Command under DAMD17-99-1-9402.

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[3] The abbreviations used are: *BRCA1*, human breast cancer 1 gene; BRCA1, protein product of *BRCA1; Brca1*, mouse breast cancer 1 gene; Brca1, protein product of *Brca1*; IR, ionizing radiation; NHEJ, nonhomologous end joining; DSB, double-strand break; MEF, mouse embryonic fibroblast; WCE, whole cell extract; mAb, monoclonal antibody; RPB1, RNA polymerase II.

by densitometry. For antibody inhibition experiments, cell extracts were preincubated with specific antibodies on ice for 30 min before shifting to 37°C for 5 min, followed by the addition of ^{32}P-labeled DNA.

Antibodies and Antisera. A recombinant protein containing glutathione *S*-transferase fused with mouse Brca1 of amino acids 788-1135 in frame was used as an antigen for the production of antimurine Brca1 mouse polyclonal antisera. Purified goat IgG specific for XRCC4, Ku70, and Ku80 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified mouse monoclonal antibody specific for BRCA1 (Ab-1) was purchased from Oncogene Research Products (San Diego, CA). Other antibodies specifically against BRCA1, Rad50, and Rad51 have been described (8).

The amounts of the following antibodies were used in NHEJ inhibition experiments: 10 μg of antimouse IgG; 1 μg of purified goat anti-Ku70 IgG; 1 μg of anti-BRCA1 mAb Ab-1; 2 μg of anti-BRCA1 mAb 17F8; 5 μg of anti-Rad50 mAb 13B3; 1 μg of purified rabbit anti-Rad51 IgG; and 1 μg of purified rabbit anti-Xrcc4. Only mAbs and commercial available purified antisera were used in antibody inhibition assays. Most of the polyclonal antisera contain high levels of nuclease activity and cannot be used in the cell-free end-joining assay.

Protein Purification. HeLa cell nuclear extract was subjected to successive phosphocellulose P-11, DEAE-Sepharose, and Superose 6 gel filtration chromatography as described previously (18). Individual fractions derived from Superose 6 chromatography corresponding to the peaks of the Rad50, Mre11, and NBS1 proteins were pooled and designated as fraction C1. The fraction C1 was concentrated on phosphocellulose P-11 by elution with 0.5 M KCl D buffer [20 mM HEPES (pH 7.9), 0.2 mM EDTA, and 20% glycerol], followed by dialysis with 0.1 M KCl in D buffer.

RESULTS

To explore the function of BRCA1 in DNA repair, we used an *in vitro* DNA end-joining assay that has been described previously (16). In this system, NHEJ catalyzed by human WCE was observed by rejoining ^{32}P-labeled linear duplex DNA in a reaction that is dependent upon all of the mammalian factors thus far genetically implicated in NHEJ, including DNA ligase IV, Xrcc4, Ku70, Ku80, and DNA-PKcs. We initially tested WCE from a human lymphoblastoid cell line, LEM, for its ability to catalyze NHEJ and observed that 25–35% of the input DNA molecules were rejoined during a 1-h incubation with 20 μg of this cell extract. Under identical reaction conditions, BRCA1-specific antibodies Ab-1 and 17F8, preincubated with LEM WCE before the addition of ^{32}P-end-labeled linear DNA into the reaction, dramatically inhibited end joining (Fig. 1). Antibodies specific for Rad50 also inhibited the end-joining activity present in WCE. Consistent with previous observations, antibodies specific for Ku70 inhibited end-joining activity *in vitro* (16). However, antibodies specific for Rad51 or normal murine IgG did not inhibit end-joining activity. These results indicate that BRCA1 and Rad50, but not Rad51, may be involved in NHEJ in this cell-free system.

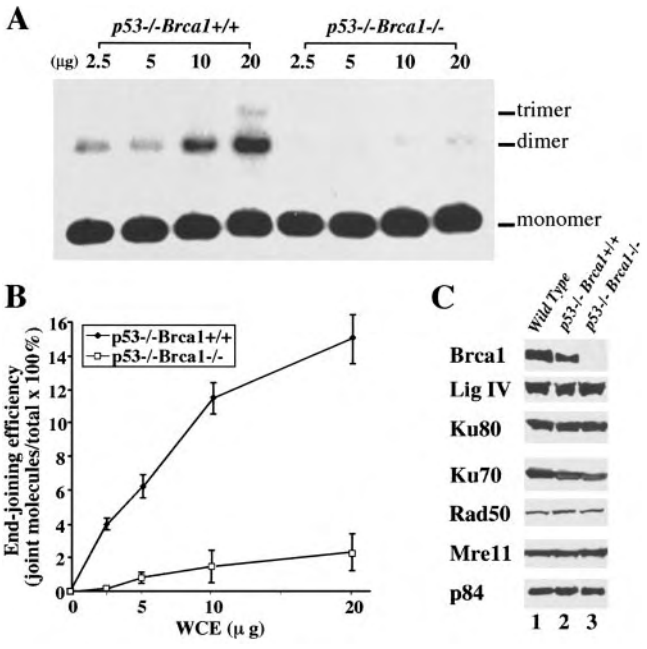

Fig. 2. Deficient end-joining activity in Brca1 mutant MEFs. *A*, end-joining activity of MEF extracts. Increasing amounts of WCE from each of the indicated MEF cell lines were incubated with ^{32}P-end-labeled linear DNA and assayed for end-joining activity. *B*, quantitative analysis by phosphorimaging. End-joining efficiency was calculated as: intensity of end-joining products/total substrate × 100%; *error bars* indicate the experimental deviation. *C*, immunoblot analysis demonstrating equivalent levels of representative NHEJ proteins and an unrelated nuclear matrix protein, p84, in the MEF WCEs assayed for end-joining activity.

To further substantiate the requirement for BRCA1 in NHEJ, we directly compared WCEs derived from *Brca1*$^{-/-}$*p53*$^{-/-}$ MEFs with extracts from both *p53*$^{-/-}$ and wild-type MEFs for their respective NHEJ activities *in vitro*. WCEs from wild-type or *p53*$^{-/-}$ MEFs could rejoin 35–50% of input DNA. Significantly, WCE from *Brca1*$^{-/-}$*p53*$^{-/-}$ MEFs was reduced 3–10-fold consistently, relative to WCE from either *p53*$^{-/-}$ or wild-type MEFs, for end-joining activity (Fig. 2, *A* and *B*). For quantitative standardization, WCEs

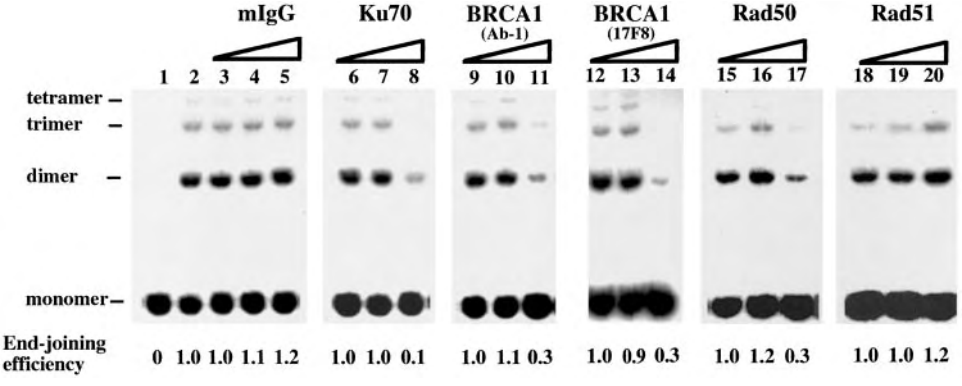

Fig. 1. Antibodies specific for BRCA1, Rad50, and Ku70 inhibit the end-joining activity present in human cell extract. Human lymphoblastoid LEM WCE was preincubated with BRCA1-specific antibodies Ab-1 (*Lanes 9–11*) and 17F8 (*Lanes 12–14*), Ku70-specific antibody (*Lanes 6–8*), Rad50-specific antibody (*Lanes 15–17*), Rad51-specific antibody (*Lanes 18–20*), or control antibody murine IgG (*Lanes 3–5*) before the addition of ^{32}P-end-labeled linear DNA (5 fmol/reaction) and subsequent incubation. Antibodies or antisera were added at 1:6 serial dilutions as follows (the actual amounts were described in the "Materials and Methods" section): straight, *Lanes 5, 8, 11, 14, 17*, and *20*; diluted 1/6, *Lanes 4, 7, 10, 13, 16*, and *19*; diluted 1/36, *Lanes 3, 6, 9, 12, 15*, and *18*. *Lane 1*: no extract. *Lane 2*: LEM extract alone. Note that BRCA1-, Rad50-, and Ku-70-specific, but not control, antibodies inhibited end joining. The end-joining efficiency was calculated as the end-joining activity [intensity of multimers/(multimers + monomer)] in each reaction expressed relative to the end-joining activity in WCEs without antibodies as determined by densitometric analysis.

were first normalized for total protein levels and subsequently analyzed by immunoblot analyses for their respective expression levels of Ku70, Ku80, DNA ligase IV, Rad50, Mre11, or the nuclear matrix protein p84 (19). No significant difference in protein levels could be observed among these WCEs (Fig. 2*C*), excluding the possibility that the reduced end-joining activity in Brca1-null cell extract is because of variations in the expression levels of these NHEJ proteins.

It is known that divalent cations such as Mg2+ and Mn2+ affect NHEJ catalyzed by mammalian WCE *in vitro* (20). Our NHEJ reactions included 0.5 mM Mg2+ and were performed identically to those described initially by Baumann *et al.* (16). To test whether the Brca1-dependent NHEJ activity present in WCEs of MEFs is affected by the concentration of divalent ions, we compared extracts derived from Brca1-deficient and wild-type MEFs for their respective NHEJ activities in the presence of increasing concentrations of Mg2+ or Mn2+. As shown in Fig. 3, augmentation of the Mg2+ or Mn2+ concentration dramatically increased the level of DNA end joining catalyzed by both Brca1-null and wild-type cell extracts and concomitantly abrogated the requirement for Brca1 regardless the amount of WCE used in the reactions. Thus, at reaction concentrations of divalent ions exceeding 1.5 mM Mg2+ or 0.5 mM Mn2+ plus 0.5 mM Mg2+, the difference in NHEJ catalyzed by Brca1-null and wild-type MEF extracts was indistinguishable, possibly indicating the involvement of a Brca1-independent pathway for NHEJ under these conditions.

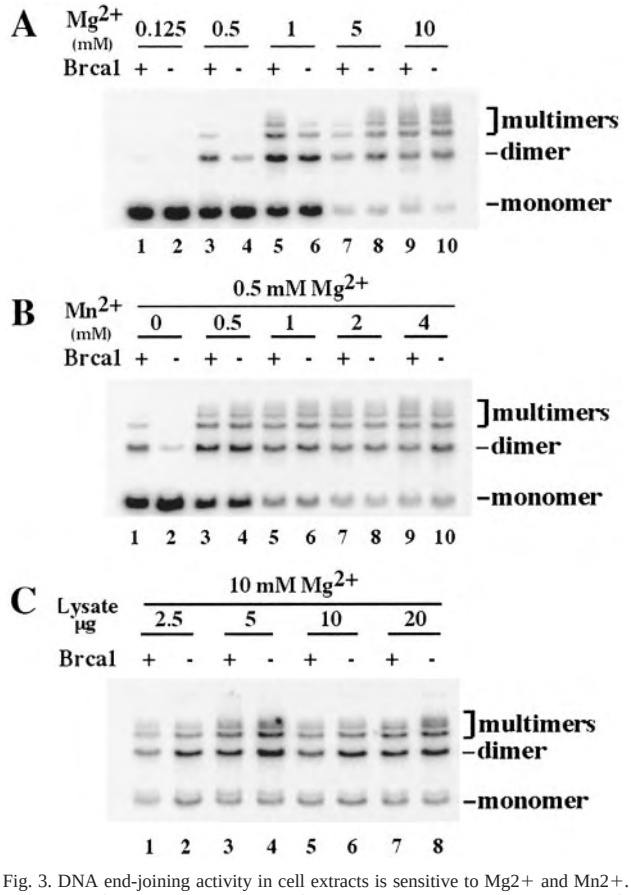

Fig. 3. DNA end-joining activity in cell extracts is sensitive to Mg2+ and Mn2+. *A*, determination of optimal concentration of Mg2+. Cell extracts (10 μg) derived from Brca1-proficient (*odd-numbered lanes*) or Brca1-deficient (*even-numbered lanes*) MEFs were incubated with different concentration of Mg2+ as follows: *Lanes 1–2*, 0.125 mM; *Lanes 3–4*, 0.5 mM; *Lanes 5–6*, 1 mM; *Lanes 7–8*, 5 mM; and *Lanes 9–10*, 10 mM. *B*, determination of the effect of Mn2+. Extract (10 μg) derived from Brca1-proficient (*odd-numbered lanes*) or Brca1-deficient (*even-numbered lanes*) MEFs were incubated at 0.5 mM Mg2+ with different concentration of Mn2+ as follows: *Lanes 1–2*, 0 mM; *Lanes 3–4*, 0.5 mM; *Lanes 5–6*, 1 mM; *Lanes 7–8*, 2 mM; and *Lanes 9–10*, 4 mM. *C*, titration of the amount of cell extracts. In the presence of 10 mM Mg2+, cell extracts (2.5, 5, 10, and 20 μg) derived from Brca1-proficient (*odd-numbered lanes*) or Brca1-deficient (*even-numbered lanes*) MEFs were assayed for DNA end-joining activity.

To explore the relationship between the Brca1-dependent NHEJ activity and known components of the NHEJ pathway, including Xrcc4 and Ku70, at different divalent ion concentrations, we performed reactions with either 0.5 mM or 10 mM Mg2+ (Fig. 4). Addition of Brca1-specific antibody reduced end-joining activity in Brca1 proficient MEFs to the level of Brca1 mutant cells at 0.5 mM Mg2+. Similarly, the addition of antibodies against Ku70 and Xrcc4 completely eliminated end-joining activity of Brca1 deficient or proficient cells. These observations suggest that BRCA1 may function along with Xrcc4 and Ku70 in NHEJ. However, at 10 mM Mg2+, the additions of antibodies against Ku70, Xrcc4, and BRCA1 have no apparent inhibitory function against the robust end-joining activities in both Brca1-proficient and -deficient MEFs (Fig. 4), indicating an existence of an alternative pathway.

To determine whether a cellular fraction containing BRCA1 can complement the diminished NHEJ activity in Brca1-deficient cells, we fractionated human HeLa cell nuclear extract according to the scheme outlined in Fig. 5*A*. The bulk of BRCA1 protein present in a soluble HeLa nuclear extract bound to phosphocellulose PC-11 and eluted predominantly and approximately equally between 0.1–0.3 and 0.3–0.5 M KCl step fractions (fractions B and C, respectively, Fig. 5*A*). The bulk of cellular Rad50 protein was also recovered in the PC-11 B and C fractions, although more eluted in the B fraction than in the C fraction (Fig. 5*A*). Most of the BRCA1 protein present in the PC-11 C fraction bound to, and eluted from, a DEAE-Sepharose anion exchange resin in a 0.1–0.25 M KCl step fraction (fraction CB). After Superose 6 gel filtration chromatography of the CB fraction, BRCA1 was eluted in fractions corresponding to peaks of the Rad50, Mre11, and Nbs1 proteins, indicating cofractionation of BRCA1 with the Rad50/Mre11/Nbs1 protein complex (Fig. 5*A*). Western blot analysis of individual Superose 6 column fractions with antibodies specific for BRCA1 and the large subunit of RPB1 also revealed that the bulk of BRCA1 eluted ahead of RPB1 in a number of high molecular weight fractions (Fig. 5*A*). Reciprocal coimmunoprecipitation of BRCA1 and Rad50 from peak Superose 6 column fractions demonstrated that BRCA1, Rad50, and Nbs1 all reside in a single high molecular weight complex of M_r ~1,000,000 (Fig. 5*B*).

Individual Superose 6 column fractions corresponding to the peak of the BRCA1/Rad50/Mre11/Nbs1 complex were pooled and concentrated on phosphocellulose PC-11 to yield a partially purified protein fraction termed C1. Fraction C1 was tested for its ability to complement WCE of Brca1-deficient MEF for end-joining activity *in vitro*. Although it catalyzed no end-joining activity on its own, the addition of fraction C1 to WCE of Brca1-deficient MEFs increased its end-joining activity about 2.5-fold (Fig. 5, *C* and *D*) to 60% of the end-joining activity catalyzed by WCE derived from Brca1-proficient MEFs. The addition of fraction C1 to WCE of Brca1-proficient MEFs has no effect on end-joining activity (data not shown). These results suggest that the partially purified BRCA1 complex facilitated the BRCA1-dependent NHEJ process.

DISCUSSION

BRCA1 plays an important role in maintaining genomic stability through its participation in DNA repair and cell cycle checkpoint control. For DNA DSB repair, BRCA1 has been shown to be critical for homologous recombination (10). However, it is not known whether BRCA1 has a role in NHEJ. In this study, we showed that under identical cell-free reaction conditions described by Baumann *et al.* (16), the addition of antibodies specific for BRCA1 and Rad50, but not Rad51, inhibits end-joining activity. Cell extracts derived from Brca1-deficient MEFs exhibit reduced end-joining activity independent of the endogenous protein amounts of DNA ligase IV, Ku80, and Ku70. The BRCA1-dependent NHEJ activity predominates at the lower concentra-

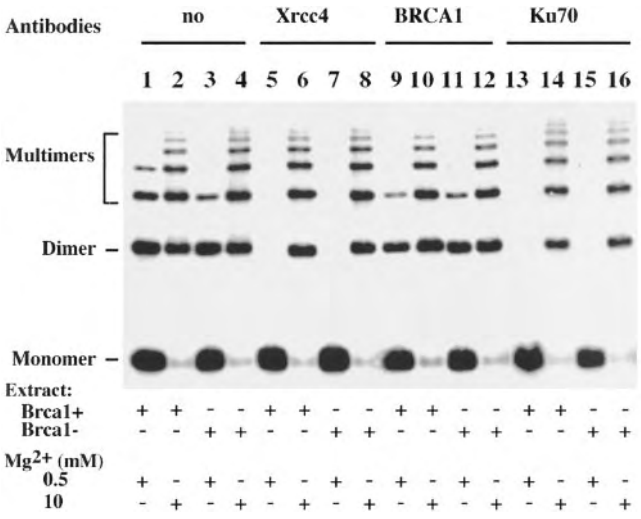

Fig. 4. Antibodies specific against BRCA1, Ku, and Xrcc4 inhibit NHEJ at 0.5 mM, but not at 10 mM, Mg2+. At 0.5 mM Mg2+ (*odd lanes*), antibodies against BRCA1 (*Lanes 9–12*) reduced the end-joining activity in Brca1-proficient MEF extracts (*Lanes 1* and *9*) to the level of Brca1-deficient cells (*Lanes 3* and *11*). Antibodies against Xrcc4 (*Lane 5–8*) or Ku70 (*Lanes 13–16*) completely inhibited the end-joining activity in Brca1-proficient cells (*Lanes 5* and *13*) and the residual activity in Brca1-deficient cells (*lanes 7* and 15). However, at 10 mM Mg2+ (*even lanes*), robust end-joining activity in both Brca1-proficient and Brca1-deficient MEFs is resistant to antibodies against Ku70 (*Lanes 6* and *8*), Xrcc4 (*Lanes 14* and *16*), and BRCA1 (*Lanes 10* and *12*), indicating an existence of an alternative pathway.

tions of 0.5 mM Mg2+; elevated reaction concentrations of Mg2+ or Mn2+ to 10 mM dramatically increases overall end-joining activity and abrogates the requirement for Brca1, Xrcc4, and Ku70. The addition of partially purified BRCA1 in association with Rad50/Mre11/Nbs1 complements the NHEJ deficiency of Brca1-deficient MEF extracts. These results support a role for BRCA1 in NHEJ.

BRCA1 is likely to participate in NHEJ by virtue of its interaction with the Rad50/Mre11/Nbs1 complex. Previous work has demonstrated that BRCA1 interacts physically with the Rad50/Mre11/Nbs1 complex *in vivo* and *in vitro* (8). Furthermore, BRCA1 can be isolated from cells in a high molecular complex with Rad50/Mre11/Nbs1. These observations suggest that the function of BRCA1 in DSB repair may be mediated, at least in part, through its association with the Rad50/Mre11/Nbs1 complex. It is recently shown that the yeast counterpart Rad50/Mre11/Xrs2 has DNA end-binding and bridging activities. Addition of the Ku homologous protein HdfA and B enhances the DNA end-bridging activity of the Rad50/Mre11/Xrs2 complex. The Rad50/Mre11/Xrs2 complex then directly recruits Dnl4/Lif1 (equivalent to mammalian DNA ligase IV and Xrcc4) to complete DNA end ligation (15). Similarly, mammalian Rad50/Mre11NBS1 has also been shown to exhibit DNA end-tethering activity (21, 22). Therefore, NHEJ *in vitro* can be carried out by components, including Rad50/Mre11/Nbs1, Ku homologues, and DNA ligase IV/Xrcc4. Although the precise role of BRCA1 in NHEJ remains unclear, the ability of BRCA1 to form a tight complex with Rad50/Mre11/Nbs1 suggests that BRCA1 may facilitate the NHEJ function of this complex. Recently, a purified recombinant BRCA1 was shown to have a direct DNA-binding activity (23). Whether this DNA-binding activity has a direct contribution to the NHEJ warrants additional investigation.

The cell-free system for NHEJ that we used in this study has been reported to be dependent on a DNA-PK-mediated pathway (16). Nonetheless, substantial evidence accumulated from *in vitro* studies indicates that eukaryotic cells rely on more than one DNA end-joining pathway (24–27). For example, extracts derived from the DNA-PK mutant cell line MO59J have been reported to exhibit wild-type end-joining activity, suggesting the involvement of a DNA-PK-independent end-joining pathway in these cells (27). The relative contribution of a particular pathway to the overall end-joining activity observed in mammalian WCEs likely

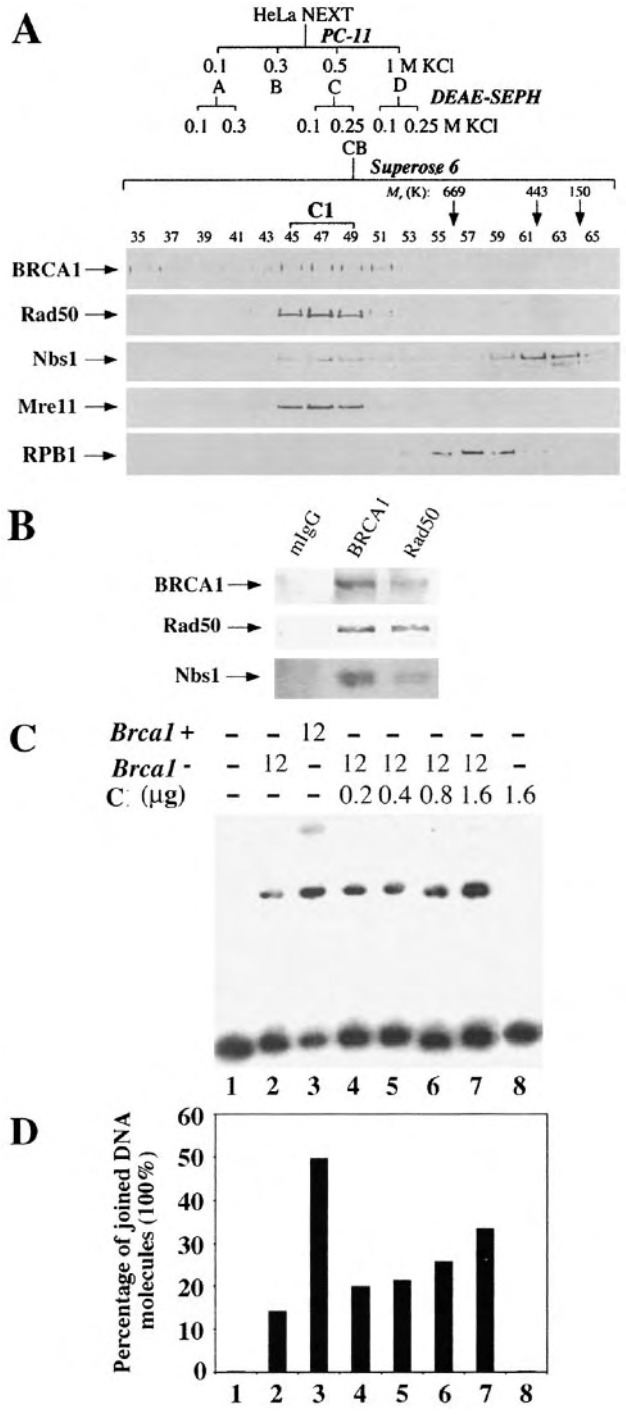

Fig. 5. Complementation of NHEJ activity in BRCA1-deficient cell extracts by exogenous BRCA1. *A*, schematic diagram of purification of a BRCA1/Rad50/Mre11/NBS1-containing complex. HeLa cell nuclear extract (NEXT) was subjected to successive phosphocellulose PC-11, DEAE-Sepharose, and Superose 6 gel filtration chromatography. CB protein fraction (4 mg/ml) was subjected to Superose 6 gel filtration analysis. Individual fractions were resolved by 10% SDS-PAGE and subjected to immunoblot analysis using antibodies specific for the proteins indicated to the *left* of the blot (RPB1: large subunit of RNA polymerase II). *Vertical arrows* above the immunoblot panels indicate marker protein peaks. *B*, coimmunoprecipitation of BRCA1, Rad50, and NBS1 from peak Superose 6 column fractions. Column fractions 45–49 (fraction C1) from the experiment in *A* were pooled, and a portion subjected to immunoprecipitation with the antibodies indicated above the immunoblot (mIgG = murine IgG). Immunoprecipitates were resolved by 10% SDS-PAGE and subjected to immunoblot analysis with antibodies specific for the proteins indicated to the left of the blot. *C*, complementation of Brca1-deficient MEF extracts for end-joining activity by a BRCA1/Rad50/Mre11/Nbs1 complex. Fraction C1 was added as indicated to Brca1-deficient WCE and incubated on ice for 30 min before assay of end-joining activity. Fraction C1 alone (*Lane 8*) does not catalyze end-joining activity. *D*, end-joining activity in *C* was measured as [intensity of multimers/(multimers + monomer) × 100%] by phosphoimage analysis and converted to percentage of joined DNA molecules.

reflects the *in vitro* reaction conditions used. One factor that could alter the relative influence of a particular end-joining pathway in the reaction is the concentration of divalent cations, particularly Mg2+ and Mn2+. In this regard, we observed that the BRCA1-dependent NHEJ activity present in mammalian WCE is sensitive to the reaction concentration of Mg2+ and Mn2+. Elevated concentrations of these divalent cations stimulates overall end-joining activity and masks the requirement for BRCA1, suggesting the involvement of a BRCA1-independent pathway to achieve end joining. A similar phenomenon has recently been reported for DNA ligase IV using ligase IV mutant 180BR cell (20). Elevated reaction concentrations of Mg2+ (10 mM) stimulated DNA end joining through an apparent DNA ligase IV-independent pathway, whereas reduced concentrations of Mg2+ (0.5 mM) revealed a DNA ligase IV dependency for low levels of end-joining activity. Interestingly, we observed that under similar conditions, antibodies specific for Ku70, Xrcc4, and BRCA1 efficiently suppressed DNA end-joining activity at reduced concentrations of Mg2+ (Fig. 4). These observations raise the possibility that BRCA1 works along with Rad50, Ku, and Xrcc4 in NHEJ at a low concentration of Mg 2+. Interestingly, mammalian cell extracts deficient in Fanconi anemia proteins had a 3–9-fold reduction in DNA end-joining activity at high Mg2+ concentration (10 mM) in a pathway independent of DNA-PK/Ku (28). Therefore, it is very likely that multiple NHEJ pathways may exist in mammalian cells.

Previously, BRCA1 has been implicated in homology-based repairs of DNA breaks because cells expressing a Brca1 exon 11-deletion mutant exhibit defects in gene targeting, single-strand annealing, and gene conversion (10). Interestingly, no defects in plasmid integration and nonhomologous repair processes were observed in these Brca1 mutant cells. However, it is possible that the NHEJ activity observed in this previous study described by Moynahan *et al*. (10), reflects the contribution of a BRCA1-independent NHEJ pathway. Alternatively, genetic differences between independently derived Brca1 mutant cell lines may contribute to different conclusions regarding the importance of Brca1 in NHEJ. Our Brca1 mutant allele carries a reverse-oriented neomycin cassette inserted into the 5′ end of Brca1 exon 11, which will lead to premature termination of translation (29). No stable Brca1 protein derivative can be detected in our Brca1$^{-/-}$ MEFs. The embryonic stem (ES) cells previously characterized for defects in homologous and NHEJ repair (10), express a Brca1 exon 11 splice variant (30) and the homozygote embryos derived from these ES cells exhibit a less severe phenotype than our homozygotes (29, 30). Using our Brcal$^{-/-}$ MEFs, we have demonstrated a 50–100-fold reduced efficiency in microhomology-mediated end-joining activity of a defined chromosomal DNA DSB introduced by a rare-cutting endonuclease I-SceI (31). These results further support a role of BRCA1 in NHEJ.

The findings presented here suggest that BRCA1 functions in the repair of DSBs through a novel role in NHEJ, consistent with the recent report that a single mutated BRCA1 allele leads to impaired fidelity of DSB end joining (32). Inefficient or error-prone DNA repair resulting directly from inactivation of BRCA1 could lead to global genomic instability and a concomitant accrual of functionally inactivating mutations at genetic loci involved in breast tumorigenesis. Additional mechanistic studies concerning the role of BRCA1 in DNA damage response and repair should expedite the design and implementation of strategies to delay, and ultimately to prevent, breast cancer formation.

ACKNOWLEDGMENTS

We thank Diane Jones and Chi-Fen Chen for excellent technical assistance.

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Appendix 5

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 8, Issue of February 20, pp. 6576–6587, 2004

Printed in U.S.A.

Functional Dissection of Transcription Factor ZBRK1 Reveals Zinc Fingers with Dual Roles in DNA-binding and BRCA1-dependent Transcriptional Repression*

Received for publication, November 10, 2003, and in revised form, November 26, 2003
Published, JBC Papers in Press, December 2, 2003, DOI 10.1074/jbc.M312270200

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The breast- and ovarian-specific tumor suppressor BRCA1 has been implicated in both activation and repression of gene transcription by virtue of its direct interaction with sequence-specific DNA-binding transcription factors. However, the mechanistic basis by which BRCA1 mediates the transcriptional activity of these regulatory proteins remains largely unknown. To clarify this issue, we have examined the functional interaction between BRCA1 and ZBRK1, a BRCA1-dependent KRAB eight zinc finger transcriptional repressor. We report here the identification and molecular characterization of a portable BRCA1-dependent transcriptional repression domain within ZBRK1 composed of zinc fingers 5–8 along with sequences in the unique ZBRK1 C terminus. This C-terminal repression domain functions in a BRCA1-, histone deacetylase-, and promoter-specific manner and is thus functionally distinguishable from the N-terminal KRAB repression domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity. Significantly, we also find that the BRCA1-dependent transcriptional repression domain on ZBRK1 includes elements that modulate its sequence-specific DNA binding activity. These findings thus reveal the presence within ZBRK1 of functionally bipartite zinc fingers with dual roles in sequence-specific DNA-binding and BRCA1-dependent transcriptional repression. We discuss the implications of these findings for the role of BRCA1 as ZBRK1 co-repressor.

Germ line inactivation of the gene encoding BRCA1 confers a cumulative lifetime risk of female breast and ovarian cancer (1–3). Although the mechanistic basis for its tissue- and gender-specific tumor suppressor activity remains poorly defined, BRCA1 nonetheless fulfills a broad function in the maintenance of global genome stability (4–10). The underlying basis for this caretaker activity likely derives from the role of BRCA1 as a conduit in the cellular DNA damage response, wherein it serves to couple DNA damage-induced signals to downstream responses including DNA damage repair and cell cycle checkpoint activation (6, 7, 11–21). Several potentially overlapping cellular activities have been ascribed to BRCA1, each of which could underlie its ability to control signal output. For example, BRCA1 has been implicated in chromatin remodeling, ubiquitinylation, recombination, and transcriptional regulation (6, 22–28). The extent to which these pleiotropic activities contribute to the caretaker function of BRCA1 is presently unknown; however, the fact that each of these BRCA1-associated activities are similarly abrogated by cancer-predisposing BRCA1 missense mutations suggests a strong correlative link between their discharge and BRCA1-mediated tumor suppression.

With respect to its role in transcription control, BRCA1 has been implicated in both activation and repression of genes linked to a variety of biological processes, including cell growth control and DNA replication and repair (21, 29–31). Thus, by virtue of its transcriptional regulatory activity, BRCA1 could influence cellular responses downstream of DNA damage signals, and this activity could contribute to its caretaker function.

The precise role of BRCA1 in gene-specific transcription control has yet to be definitively established. Because it exhibits no sequence-specific DNA binding activity, it seems likely that BRCA1 is targeted to specific genes through its functional interaction with sequence-specific DNA-binding transcription factors. Direct evidence to support this hypothesis has come from the identification of multiple DNA-binding transcription factors with which BRCA1 has been shown to physically interact and functionally synergize, including p53, c-Myc, estrogen receptor α, androgen receptor, OCT-1, NF-YA, and ZBRK1 (32–40). However, the underlying mechanism by which BRCA1 mediates the transcriptional stimulatory or repressive effects of these regulatory proteins has not been established.

We have been studying the functional interaction between BRCA1 and the transcriptional repressor ZBRK1 as a model system to understand the mechanistic basis by which BRCA1 mediates sequence-specific transcription control. Initially identified by virtue of its physical interaction with BRCA1, ZBRK1 (Zinc finger and BRCA1-interacting protein with a KRAB domain 1) is a member of the Kruppel-associated box-zinc finger protein (KRAB-ZFP) family of transcriptional repressors (39, 41). Typically, KRAB-ZFPs bind to their corresponding target genes through tandem C-terminal C_2H_2 zinc fingers and repress transcription through an N-terminal KRAB domain, which silences gene expression through the indirect recruitment of histone deacetylases, histone methyltransferases, and heterochromatin proteins (41–47). Like other KRAB-ZFPs, ZBRK1 harbors an N-terminal KRAB domain. However, ZBRK1 is atypical among KRAB-ZFPs due to the fact that it harbors 8 central C_2H_2 zinc fingers and a unique C terminus

* This work was supported by United States Army Department of Defense Grant DAMD17-02-1-0584 (to T. G. B.), United States Army Department of Defense Training Grant (to W. T.), and National Institutes of Health Grants CA94170, CA81020 (to W.-H. L.), and CA098301-01 (to T. G. B). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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that is absent among the larger family of KRAB-ZFPs.

Through its 8 central zinc fingers, ZBRK1 binds to a compositionally flexible 15-bp DNA sequence, GGG*xxx*CAG*xxx*TTT (where *x* is any nucleotide) (39). A search for potential ZBRK1 DNA-binding sites in existing genes led to intron 3 of GADD45a, a functionally important DNA damage-response effector known to be regulated transcriptionally by BRCA1 (21, 32, 39). Functional analysis revealed that ZBRK1 represses *GADD45a* gene transcription through its intron 3 DNA-binding site in a BRCA1-dependent manner, thus revealing BRCA1 to be a ZBRK1 co-repressor (39). Significantly, familial breast cancer-derived mutants of BRCA1 that disrupt its interaction with ZBRK1 abrogate its co-repressor activity, suggesting that its co-repressor function may be important for the tumor suppressor properties of BRCA1 (39). The regulation of *GADD45a* gene transcription is likely to be complex and controlled coordinately by ZBRK1 and BRCA1 in concert with other transacting factors, including p53, OCT1, and NF-YA, that function through cis-acting sequences present in the *GADD45a* promoter and intron 3 regions (21, 32, 39, 48, 49).

In addition to *GADD45a*, potential ZBRK1-binding sites have been identified in other DNA damage-response genes that are also regulated by BRCA1, including p21, Bax, and GADD153 (39). This observation suggests a potentially broader role for BRCA1 and ZBRK1 in the coordinate transcriptional regulation of diverse DNA damage-response genes. To begin to explore the mechanism by which BRCA1 mediates sequence-specific transcriptional repression through ZBRK1, we have pursued in greater depth the physical and functional interaction between these two proteins. We report here the identification and molecular characterization of a portable BRCA1-dependent transcriptional repression domain within ZBRK1 composed of zinc fingers 5–8 along with sequences in the unique ZBRK1 C terminus. This C-terminal repression domain functions in a BRCA1-, histone deacetylase (HDAC)-,[1] and promoter-specific manner and is thus functionally distinguishable from the N-terminal KRAB repression domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity. Significantly, we also find that the BRCA1-dependent C-terminal transcriptional repression domain within ZBRK1 is composed of elements that modulate sequence-specific DNA-binding by zinc fingers 1–4. These findings thus reveal an unanticipated dual function for the ZBRK1 zinc fingers in DNA binding and transcriptional repression, and further shed new light on the mechanistic role of BRCA1 in sequence-specific transcriptional control.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis

Expression Plasmids—pMAL-C2-TEV-ZBRK1 ΔK for expressing MBP-ZBRK1 ΔK in *Escherichia coli* was derived from pGEPK3-ZBRK1 ΔK, which is a derivative of pCNF-ZBRK1 ΔK (39). Briefly, a BamHI-XhoI fragment carrying ZBRK1 cDNA sequences encoding ZBRK1 amino acids 144–532 (lacking the N-terminal KRAB domain) was subcloned into the BamHI and XhoI sites of pMAL-C2-TEV (provided by Dr. P. Renee Yew), thereby generating a translational fusion of MBP and ZBRK1 ΔK. C-terminal truncation mutants of MBP-ZBRK1 ΔK bearing stepwise deletions of individual ZBRK1 zinc fingers were expressed from pMAL-C2-TEV-ZBRK1 ΔK deletion derivatives, each of which was constructed by PCR-based subcloning. Briefly, sequences encoding ZBRK1 ΔK within pMAL-C2-TEV-ZBRK1 ΔK were liberated as a BamHI-HindIII fragment and replaced with corresponding PCR-generated ZBRK1 deletion fragments using a common upstream primer corresponding to MBP sequences and unique downstream primers within the C terminus of each zinc finger (defined here as the seventh amino acid residue C-terminal to the last histidine residue of each C_2H_2 zinc finger). Individual MBP-ZBRK1 ΔK deletion derivatives encode the following ZBRK1 amino acids (aa): MBP-ZBRK1 ΔK 8ZF (aa 144–431); 7ZF (aa 144–403); 6ZF (aa 144–377); 5ZF (aa 144–347); 4ZF (aa 144–319); 3ZF (aa 144–291); 2ZF (aa 144–263); and 1ZF (aa 144–235). Individual MBP-ZBRK1 ΔK broken finger (BF) mutants were generated by PCR-based site-directed mutagenesis of pMAL-C2-TEV-ZBRK1 ΔK using the QuickChange II site-directed mutagenesis kit following the manufacturer's recommendations (Stratagene, La Jolla, CA). Each broken finger mutant bears a histidine (CAT codon) to asparagine (aaT codon) substitution mutation at the first of the two conserved histidine residues within the targeted C_2H_2 zinc finger.

ZBRK1 5ZFC was expressed as a GAL4 DNA-binding domain fusion in mammalian cells from the plasmid GAL4-ZBRK1 5ZFC, constructed by subcloning a PCR-amplified ZBRK1 cDNA fragment encoding amino acids 319–532 (encompassing zinc finger 5 through the C terminus) into the SalI and HindIII sites of pM (Clontech, Palo Alto, CA). N- and C-terminal truncation derivatives of GAL4-ZBRK1 5ZFC bearing stepwise deletions of individual zinc fingers and C-terminal sequences, respectively, were generated by PCR-based subcloning. Briefly, the SalI-HindIII fragment within GAL4-ZBRK1 5ZFC encoding ZBRK1 5ZFC was replaced with corresponding PCR-generated ZBRK1 deletion fragments. Individual GAL4-ZBRK1 5ZFC deletion derivatives encode the following ZBRK1 amino acids (aa): GAL4-ZBRK1 6ZFC (aa 347–532); 7ZFC (aa 375–532); 8ZFC (aa 403–532); C (aa 431–532); GAL4-ZBRK1 5ZFC Δ1 (aa 319–523); 5ZFC Δ2 (aa 319–503); and 5ZFC Δ3 (aa 319–483). Broken finger derivatives of GAL4-ZBRK1 5ZFC were generated by PCR amplification of ZBRK1 sequences encoding amino acids 319–532 from individual pMAL-C2-TEV ZBRK1 ΔK BF mutants and subsequent replacement of the SalI-HindIII wild-type ZBRK1 fragment in GAL4-ZBRK1 5ZFC.

ZBRK1 5ZFC and its truncation and broken finger derivatives were expressed in yeast as GAL4 activation domain fusions using pGADT7 (Clontech, Palo Alto, CA). Briefly, ZBRK1 5ZFC and its truncation (6ZFC, 7ZFC, 8ZFC, 0ZFC, 5ZFC Δ1, 5ZFC Δ2, and 5ZFC Δ3) and broken finger (BF5, BF6, BF7, and BF8) derivatives were excised as BamHI-blunted SalI fragments from GAL4-ZBRK1 5ZFC and its corresponding derivative plasmids, and subcloned into the BamHI and blunted XhoI sites in pGADT7. A BRCA1 cDNA fragment (encoding amino acids 341–748) encompassing the ZBRK1-binding domain (39) was PCR-amplified and subcloned into the EcoRI and SalI sites of pGBKT7, thereby generating a translational fusion of the GAL4 DNA-binding domain with BRCA1 amino acids 341–748 in the plasmid pGBKT7-BRCA1.

The ZBRK1 KRAB domain was expressed as a GAL4 DNA-binding domain fusion in mammalian cells from GAL4-ZBRK1 KRAB, constructed by subcloning a PCR-amplified ZBRK1 cDNA fragment encoding amino acids 1–85 into the BamHI and blunted XbaI sites of pM2 (50). All PCR-based subcloning was performed using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), and the integrity of individual deletion and substitution mutations was confirmed by DNA sequence analysis.

Reporter Plasmids—pG_5TK-Luc carrying five copies of the GAL4 DNA-binding site upstream of the herpes simplex virus thymidine kinase (TK) promoter (sequences corresponding to −105 to +51, where +1 is the transcription start site) driving expression of the gene encoding firefly luciferase was constructed by replacing a HindIII-BglII fragment from pSBS-GAL-TK-Luc (provided by Dr. Tony Ip) with a HindIII-BglII fragment from pG_5TK-CAT (provided by Dr. P. Renee Yew), thus positioning five copies of the GAL4 DNA-binding site and the TK promoter upstream of the firefly luciferase gene. pG_5SV40-Luc carrying five GAL4 DNA-binding sites upstream of the SV40 promoter driving expression of the firefly luciferase gene has been described previously (39). pG_5SNRPN-Luc (provided by Dr. Paul A. Wade) carries five GAL4 DNA-binding sites upstream of the human small nuclear ribonucleoprotein N promoter driving expression of the firefly luciferase gene (51).

Recombinant Protein Expression and Purification

MBP-ZBRK1 fusion proteins were expressed in and purified from *E. coli* strain BL21 Star (DE3) pLysS (Invitrogen). Briefly, cells were grown at 37 °C to an A_{600} of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration 0.3 mM, and the cells were transferred to 25 °C for another 3.5 h. Cells were pelleted, washed once with phosphate-buffered saline, and then resuspended in MBP binding buffer (25 mM Tris-HCl, pH 7.5; 1 mM EDTA; 200 mM NaCl; 20 μM

[1] The abbreviations used are: HDAC, histone deacetylase; EMSA, electrophoretic mobility shift assay; MEFs, mouse embryo fibroblasts; aa, amino acids; MBP, maltose-binding protein; PMSF, phenylmethylsulfonyl fluoride; TSA, trichostatin A; ZRE, ZBRK1-response element; BF, broken finger; TK, thymidine kinase; WT, wild type; Mut., mutant; HSV, herpes simplex virus.

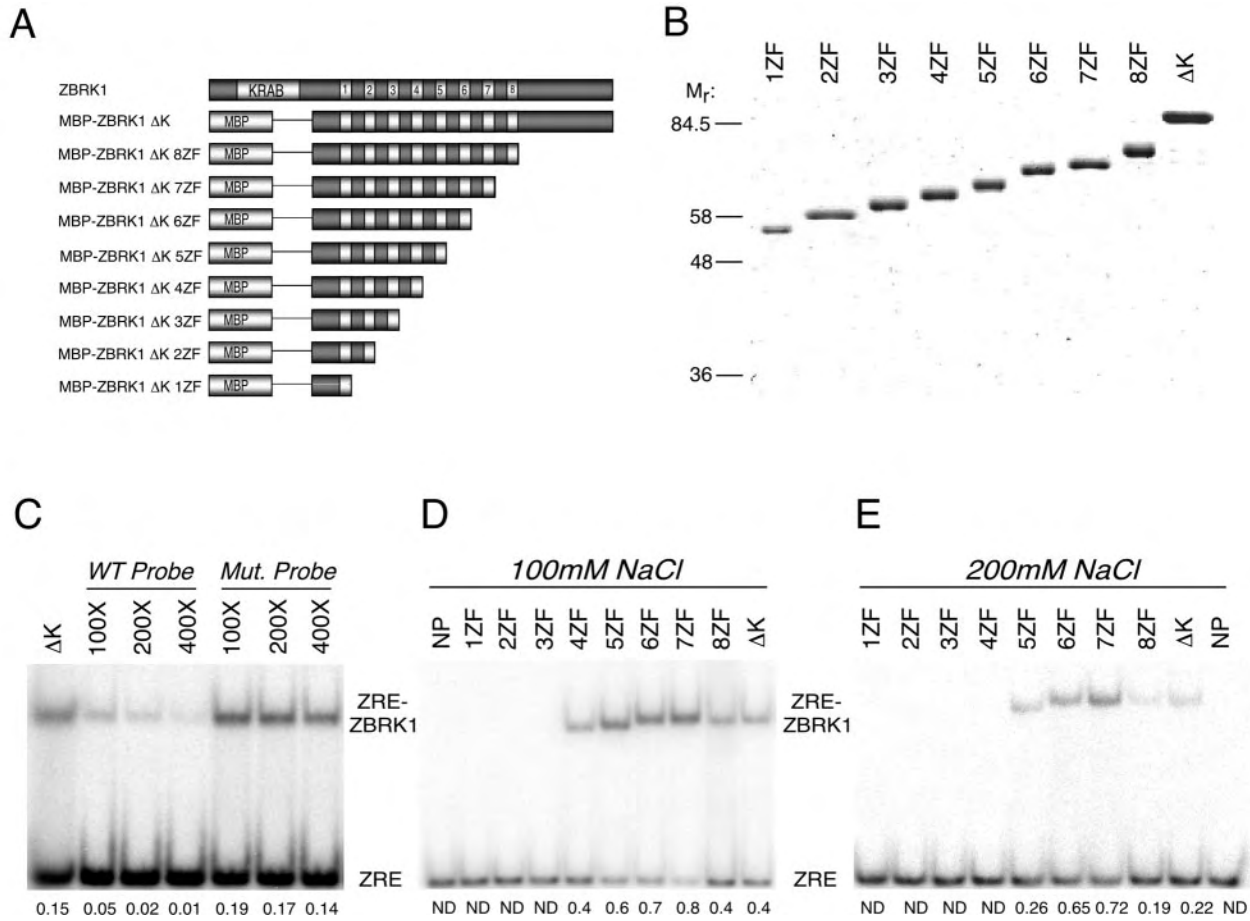

FIG. 1. **Identification of DNA binding determinants on ZBRK1.** *A*, schematic representation of ZBRK1 (the KRAB domain and numbered zinc fingers are indicated), MBP-ZBRK1 ΔK, and MBP-ZBRK1 ΔK truncation derivatives. *B*, purified MBP-ZBRK1 ΔK and its corresponding truncation derivatives as indicated were resolved by SDS-10% PAGE and visualized by Coomassie Blue staining. Molecular weight marker positions (M_r) are indicated. *C*, competition EMSA. EMSA was performed using a ^{32}P-labeled double-stranded oligonucleotide probe corresponding to a wild-type consensus ZRE and purified MBP-ZBRK1 ΔK (*1st lane*). A 100-, 200-, or 400-fold molar excess of an unlabeled wild-type (*WT*) ZRE probe (*2nd to 4th lanes*) or mutated (*Mut*.) ZRE probe corresponding to a double-stranded oligonucleotide identical in length but different in sequence (*5th to 7th lanes*) was added to the binding reaction as indicated. The positions of the unbound ZRE oligonucleotide probe (*ZRE*) and the ZRE·MBP-ZBRK1 ΔK nucleoprotein complex (*ZRE-ZBRK1*) are indicated. For each EMSA reaction, the proportional fraction of ZRE probe bound by MBP-ZBRK1 ΔK is indicated *below* each lane and was determined by dividing the number of radioactive counts in the bound probe by the number of radioactive counts in the bound plus the unbound probe. *D* and *E*, sequence-specific DNA binding activity of MBP-ZBRK1 ΔK and its corresponding truncation derivatives. EMSA was performed using the consensus ZRE probe and 50 ng of either MBP-ZBRK1 ΔK or each of its corresponding deletion derivatives as indicated. *NP* indicates no protein added to the EMSA reaction. The positions of the unbound ZRE oligonucleotide probe (*ZRE*) and the ZRE·MBP-ZBRK1 ΔK nucleoprotein complex (*ZRE-ZBRK1*) are indicated. DNA-binding reactions were performed in 100 mM NaCl (*D*) or 200 mM NaCl (*E*). For each EMSA reaction, the proportional fraction of ZRE probe bound by MBP-ZBRK1 ΔK or its truncation derivatives is indicated *below* each lane and was determined by dividing the number of radioactive counts in the bound probe by the number of radioactive counts in the bound plus the unbound probe. *ND*, not detectable.

$ZnCl_2$; and 10 mM β-mercaptoethanol) supplemented with protease inhibitors (aprotinin 0.4 μg/ml; chymostatin 0.5 μg/ml; leupeptin 0.5 μg/ml; pepstatin 0.5 μg/ml; PMSF 0.5 mM; and benzamidine-HCl 0.5 mM). Resuspended cells were frozen and thawed one time, followed by sonication (3 times for 1 min) and clarification by centrifugation at 30,000 × *g* for 30 min. MBP-ZBRK1 fusion proteins were purified from clarified lysates by affinity chromatography on amylose resin (New England Biolabs, Beverly, MA). Briefly, clarified lysates were incubated with amylose resin in batch for 1 h at 4 °C, washed 3 times with MBP binding buffer, and then eluted with MBP binding buffer containing 0.5% maltose for 30 min at 4 °C. Purified proteins (estimated to be >95% homogeneous by SDS-PAGE and subsequent visualization by Coomassie Blue staining) were dialyzed for 1 h at 4 °C against EMSA storage buffer (25 mM Tris-HCl, pH 7.5; 100 mM NaCl; 20 μM $ZnCl_2$; 10% glycerol; 10 mM β-mercaptoethanol; and 0.5 mM PMSF) before long term storage at −80 °C.

Electrophretic Mobility Shift Assay (EMSA)

For EMSA, the ZRE probe was obtained by annealing two complementary oligonucleotides corresponding in sequence to the consensus ZBRK1 DNA-binding site: 5′-GATCCACGGGACGCAGGTGTTTTGTGCCG-3′ and 5′-GATCCGGCACAAAACACCTGCGTCCCGTG-3′ (39). Mutant (Mut) probe was obtained by annealing two oligonucleotides, 5′-GATCCACCTCACGTTCGTGCACTGTGCCG-3′ and 5′-GATCCGGCACAGTGCACGAACGTGAGGTG-3′ (39). Each of these double-stranded probes carried overhanging ends, which were filled in with [α-^{32}P]dCTP by Klenow enzyme. In each reaction, purified MBP-ZBRK1 fusion proteins (50 ng) were incubated with 6000 cpm of a ^{32}P-labeled double-stranded oligonucleotide probe in 30 μl of EMSA binding buffer (25 mM Tris-HCl pH 7.5; 20 μM $ZnCl_2$; 12.5% glycerol; 0.5 mM PMSF; and a variable concentration of NaCl as indicated). Following 30 min of incubation at room temperature, reaction mixtures were loaded onto a 5% non-denaturing polyacrylamide gel and electrophoresed at 200 V for 2 h at 4 °C in 0.5× TBE. Dried gels were subjected to PhosphorImager analysis (Amersham Biosciences).

Cell Culture, Transfections, and Reporter Assays

Brca1−/−, p53−/− (*Brca1*−/−), and p53−/− (*Brca1*+/+) mouse embryo fibroblasts (MEFs) (39) and U2OS human osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). *Brca1*+/+, *Brca1*−/−, and U2OS cells were transfected at 60% confluency using Effectene reagent (Qiagen, Valencia, CA), and the expression and reporter plasmids are indicated in each figure. Each transfection also included an internal control plasmid, pCH110 (40), expressing β-galactosidase under control of the SV40 promoter. Forty-eight hours

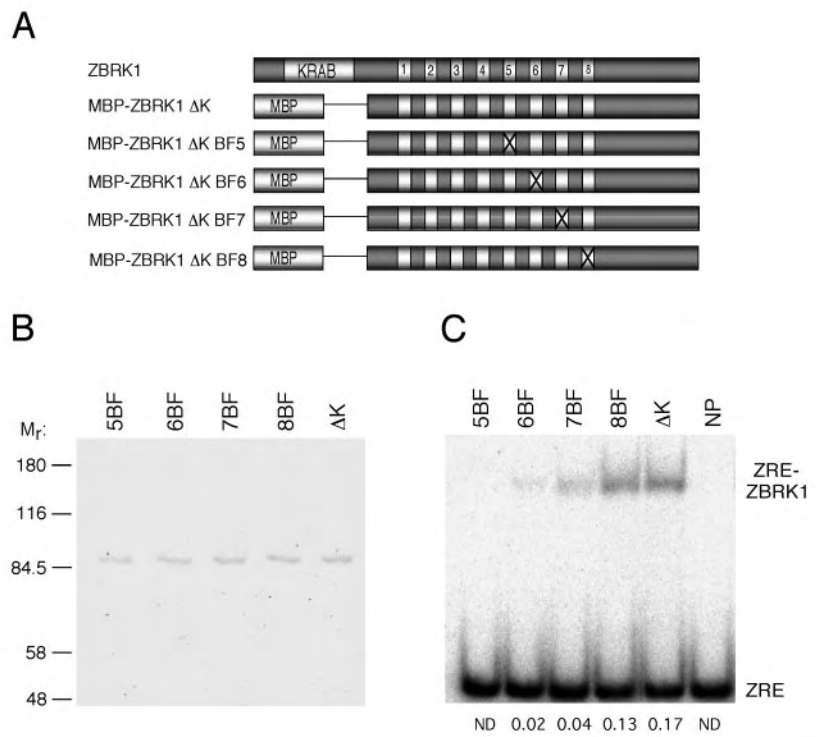

FIG. 2. **Identification of DNA-binding determinants on ZBRK1.** *A*, schematic representation of ZBRK1, MBP-ZBRK1 ΔK, and MBP-ZBRK1 ΔK broken finger derivatives. *B*, purified MBP-ZBRK1 ΔK and its corresponding broken finger derivatives as indicated were resolved by SDS-10% PAGE and visualized by Coomassie Blue staining. Molecular weight marker positions (M_r) are indicated. *C*, sequence-specific DNA binding activity of MBP-ZBRK1 ΔK and its corresponding broken finger derivatives. EMSA was performed using the consensus ZRE probe and 50 ng of either MBP-ZBRK1 ΔK or each of its corresponding broken finger derivatives as indicated. *NP* indicates no protein added to the EMSA reaction. The positions of the unbound ZRE oligonucleotide probe (*ZRE*) and the ZRE·MBP-ZBRK1 ΔK nucleoprotein complex (*ZRE-ZBRK1*) are indicated. DNA-binding reactions were performed in 200 mM NaCl. For each EMSA reaction, the proportional fraction of ZRE probe bound by MBP-ZBRK1 ΔK or its broken finger derivatives is indicated *below* each lane and was determined by dividing the number of radioactive counts in the bound probe by the number of radioactive counts in the bound plus the unbound probe. *ND*, not detectable.

post-transfection, cells were harvested and lysed in Reporter Lysis buffer (Promega, Madison, WI). Transfected cell lysates (20 μl) were analyzed for luciferase activity using the luciferase assay system (Promega, Madison, WI) and for β-galactosidase activity using the Galactolight Plus Chemiluminescent Reporter Assay (BD Biosciences). Each transfection was repeated a minimum of 3 times in duplicate.

Yeast Two-hybrid Interaction Assay

pGADT7-ZBRK1 5ZFC and its truncation (6ZFC, 7ZFC, 8ZFC, 0ZFC, 5ZFC Δ1, 5ZFC Δ2, and 5ZFC Δ3) and broken finger (BF5, BF6, BF7, and BF8) derivatives were individually co-transformed along with pGBKT7-BRCA1 (expressing BRCA1 amino acids 341–748) into the yeast strain Y187 (BD Biosciences/Clontech). After selection, colonies were expanded in liquid culture for assay of β-galactosidase activity following previously established procedures (39).

Transient Expression Analysis

Steady-state levels of GAL4-ZBRK1 5ZFC protein and its truncation and substitution derivatives were comparatively analyzed by immunoblot analysis of transfected whole cell extracts in order to verify equivalent levels of ectopic protein expression. Briefly, U2OS cells transfected with GAL4-ZBRK1 5ZFC, truncation mutants GAL4-ZBRK1 6ZF, 7ZFC, 8ZFC, 0ZFC, 5ZFC Δ1, 5ZFC Δ2, and 5ZFC Δ3, and broken finger mutants GAL4-ZBRK1 5ZFC BF5, BF6, BF7, and BF8 were lysed in Laemmli sample buffer, resolved by SDS-10% PAGE, and subjected to immunoblot analysis using an antibody directed against the GAL4 DNA-binding domain (sc-510, Santa Cruz Biotechnology, Santa Cruz, CA) and, as an internal control protein, the p89 subunit of TFIIH (sc-293, Santa Cruz Biotechnology, Santa Cruz, CA). Immunodetection was performed using ECL Western blotting detection reagents (Amersham Biosciences).

RESULTS

Identification of DNA-binding Determinants on ZBRK1—Previously, we demonstrated a strict requirement for BRCA1 in ZBRK1 repression. Specifically, we showed that ZBRK1 repression function was similarly abrogated by genetic ablation of *Brca1* or by deletion of the BRCA1-binding domain on ZBRK1 (39). The BRCA1-binding domain on ZBRK1 includes the last four of eight ZBRK1 zinc fingers (zinc fingers 5–8) along with the ZBRK1 C terminus (39). Whether and how this zinc finger domain contributes to the sequence-specific DNA binding activity of ZBRK1, however, is presently unknown. Because the specification of individual zinc fingers required to bind to DNA and/or BRCA1 could illuminate the underlying mechanism(s) by which BRCA1 mediates ZBRK1 repression, we initially sought to establish both the number and identity of the ZBRK1 zinc fingers required to bind DNA and BRCA1.

With respect to DNA binding, we showed previously (39) that the eight central ZBRK1 zinc fingers collectively recognize a 15-bp consensus sequence, GGG*xxx*CAG*xxx*TTT. Based on the observation that one C_2H_2 zinc finger can bind to ~3 bp of DNA (52–54), only five of the eight ZBRK1 zinc fingers would be predicted to bind to its 15-bp consensus sequence. To test this prediction, we analyzed a panel of ZBRK1 zinc finger deletion derivatives for their respective abilities to bind to the consensus ZBRK1 DNA-binding site in an EMSA.

To this end, we expressed ZBRK1 as a maltose-binding protein (MBP) chimera in *E. coli*, which permitted the purification of otherwise insoluble ZBRK1 protein. Full-length MBP-ZBRK1 is expressed poorly, whereas MBP-ZBRK1 ΔK (a deletion derivative lacking the N-terminal 143 amino acids of the 532-amino acid full-length ZBRK1 protein) is abundantly expressed. ZBRK1 ΔK lacks the N-terminal KRAB domain but retains the ZBRK1 zinc fingers and the C terminus, elements that are required for binding to both DNA and/or BRCA1 (Fig. 1, *A* and *B*). We have therefore utilized this recombinant ZBRK1 derivative as the background into which truncation and substitution mutations have been introduced for purposes of DNA binding assays.

In an EMSA, MBP-ZBRK1 ΔK produced a discrete nucleoprotein complex on a double-stranded oligonucleotide probe corresponding to the consensus ZBRK1-response element (ZRE); a molar excess of unlabeled WT ZRE probe (WT probe), but not a mutant probe (Mut probe), efficiently competed for the formation of this complex, thus establishing sequence-specific DNA binding by MBP-ZBRK1 ΔK in this assay (Fig. 1*C*). To determine the number and identity of the ZBRK1 zinc fingers required to bind to its consensus sequence, we analyzed a series of C-terminal truncation derivatives bearing stepwise deletions of individual ZBRK1 zinc fingers (Fig. 1, *A* and *B*). In

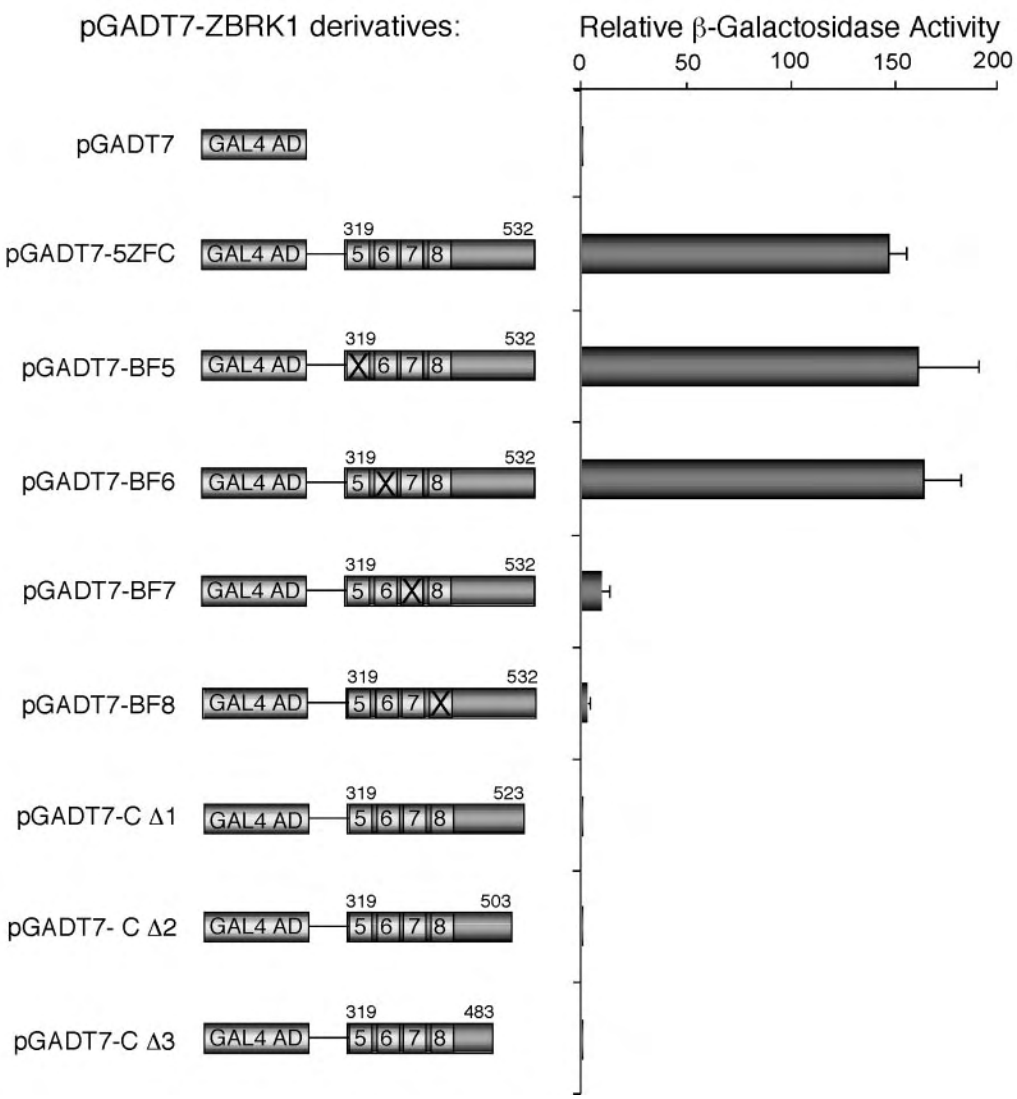

FIG. 3. **Identification of BRCA1-binding determinants on ZBRK1.** The ZBRK1-binding region on BRCA1 (amino acids 341–748) (39), expressed as a GAL4 DNA-binding domain fusion protein (in the plasmid pGBKT7), was tested for interaction with the indicated fragments of ZBRK1 fused to the GAL4 transactivation domain (plasmid pGADT7) in yeast two-hybrid assays. β-Galactosidase activities were quantified as described previously (39). Corresponding β-galactosidase activities obtained with each pGADT7-ZBRK1 5ZFC derivative are expressed relative to that observed with the backbone pGADT7 expression vector alone, which was arbitrarily assigned a value of 1. Values represents the average of three independent assays, each performed in triplicate, and *error bars* represent the mean ± S.D. As a comparative measure of interaction strength, the average β-galactosidase activity obtained with pGBKT7-p53 and pGADT7-Large T antigen was 512 ± 13.5. The validity of two-hybrid interactions observed in these experiments was further substantiated by the following controls. First, we confirmed that the observed β-galactosidase activities were dependent upon BRCA1 sequences expressed from the plasmid pGBKT7-BRCA1 by performing a parallel series of interaction assays with individual pGADT7-ZBRK1 5ZFC derivatives and the backbone vector pGBKT7 as a negative control. Second, immunoblot analyses of yeast whole cell extracts confirmed that each of the pGADT7-ZBRK1 5ZFC fusion proteins was expressed at roughly equivalent levels, thus excluding the possibility that differences in β-galactosidase activities derive from difference in fusion protein expression.

100 mM NaCl, truncation of the ZBRK1 C terminus (MBP-ZBRK1 ΔK 8ZF) did not appreciably affect DNA binding relative to intact MBP-ZBRK1 ΔK (Fig. 1*D*). Interestingly, deletion of the eighth and last ZBRK1 zinc finger along with the C terminus (MBP-ZBRK1 ΔK 7ZF) led to an increase in DNA binding activity, suggesting that ZBRK1 ZF8, and possibly the C terminus, constrains sequence-specific DNA binding mediated by the first seven zinc fingers (Fig. 1*D*). This effect was exacerbated under more stringent DNA binding conditions (200 mM NaCl) (Fig. 1*E*). At 100 mM NaCl, stepwise truncation of zinc fingers 7 to 5 led to a slight incremental reduction in sequence-specific DNA binding activity (Fig. 1*D*). ZBRK1 derivatives bearing less than four zinc fingers failed to bind to DNA, thereby establishing zinc fingers 1–4 as the minimal ZBRK1 DNA-binding domain under these conditions (Fig. 1*D*). Identical results were observed at 50 mM NaCl (data not shown). At 200 mM NaCl, zinc fingers 1–5 were required for stable DNA binding, and the inclusion of zinc fingers 6 and 7 incrementally stabilized binding (Fig. 1*E*).

To examine more rigorously the role of zinc fingers 5–8 in sequence-specific DNA binding by ZBRK1, we examined a set of "broken finger" mutants bearing substitution mutations within each of these zinc fingers. This approach permitted us to assess the individual contribution of each finger within the BRCA1-binding domain to overall DNA binding activity in the context of the eight-fingered ZBRK1 ΔK protein and thereby circumvent potential artifacts arising from analyses of truncation mutants. Each broken finger mutant bears a His-to-Asn substitution mutation at the first of the two conserved His residues within the targeted C_2H_2 zinc finger (Fig. 2, *A* and *B*). The relative conservative nature of this substitution eliminates zinc coordination within the targeted finger, thereby disrupting

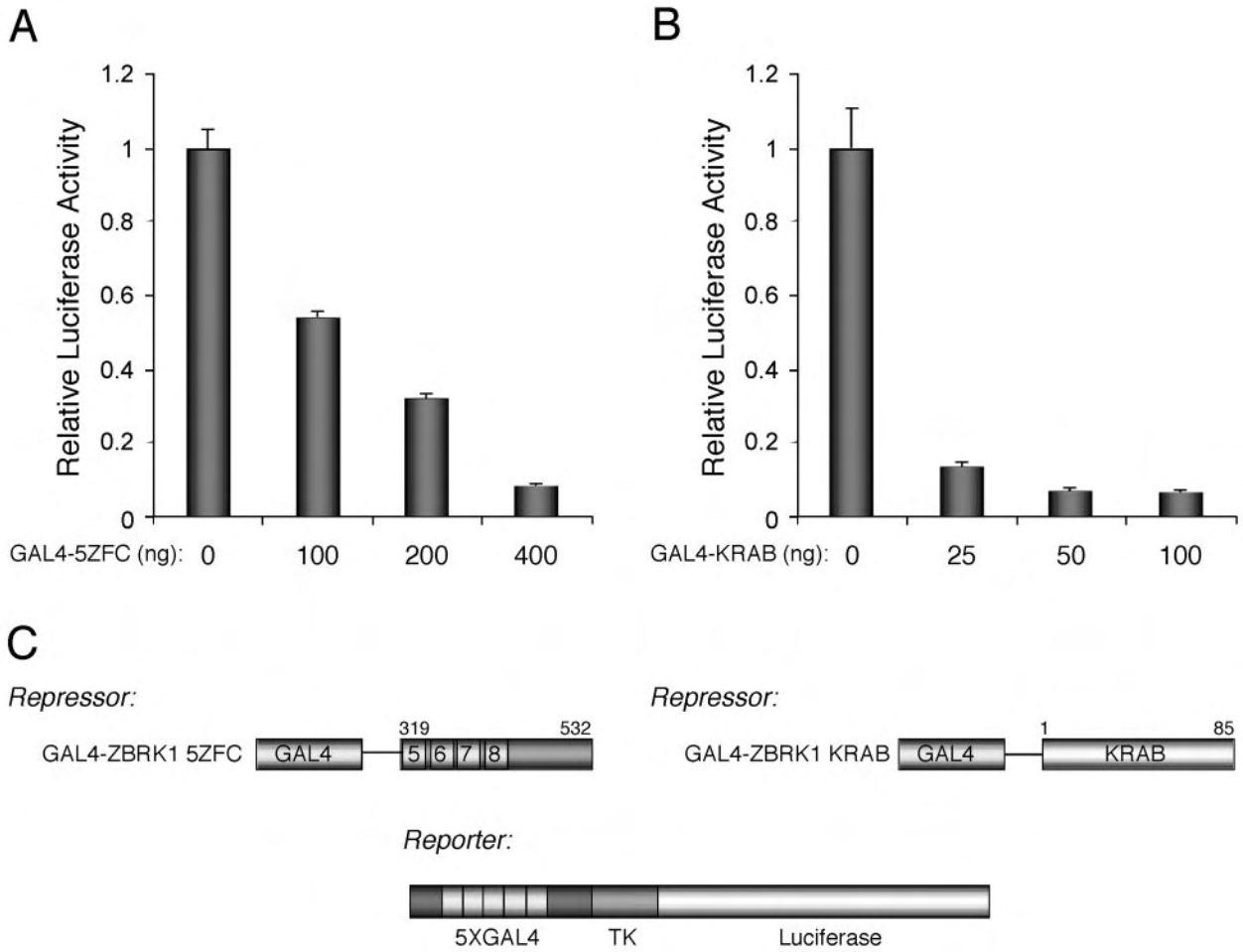

FIG. 4. **ZBRK1 harbors two independent transcriptional repression domains.** *A* and *B*, human U2OS cells were transfected with 30 ng of pG_5TK-Luc bearing five copies of the GAL4 DNA-binding site sequence upstream of the TK promoter without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC (*A*) or GAL4-KRAB (*B*). In this and all subsequent transfection experiments involving effector plasmid titrations, the total amount of DNA in each transfection was fixed by reciprocal titration of the corresponding backbone expression plasmid. Also, in this and all subsequent transfection experiments, the relative luciferase activity represents the ratio of the luciferase activity obtained in a particular transfection to that obtained in cells transfected with only the reporter and pM (GAL4 DNA-binding domain) expression vectors alone. Luciferase activities were first normalized to β-galactosidase activity obtained by co-transfection of the SV40-β-gal vector (15 ng) as described previously (39). *Error bars* represent the S.D. from the average of at least three independent transfections performed in duplicate. *C*, schematic representation of GAL4-ZBRK1 5ZFC and GAL4-ZBRK1 KRAB chimeras (amino acid sequences fused to the GAL4 DNA-binding domain are indicated *numerically above* each chimera) and the pG_5TK-Luc reporter template used in transfection assays.

its local structure with little effect on the integrity of the remainder of the protein (55, 56). Consistent with the results obtained using C-terminal truncation mutants, analysis of individual BF mutants 5–8 revealed zinc finger 5 to be an important ZBRK1 determinant for stable DNA binding, whereas zinc fingers 6 and 7 promote but are not essential for binding (Fig. 2*C*). Disruption of zinc finger 8 did not appreciably affect the DNA binding activity of MBP-ZBRK1 ΔK, suggesting that zinc finger 8 is largely dispensable for stable association with DNA (Fig. 2*C*). Furthermore, local disruption of zinc finger 8 did not relieve constraints on the DNA binding activity of MBP-ZBRK1 ΔK, suggesting that the C terminus of ZBRK1 can also mask the inherent DNA binding activity of ZBRK1 zinc fingers 1–7 (Fig. 2*C*). In summary, the results of DNA-binding analyses delimit the core ZBRK1 DNA-binding domain to zinc fingers 1–4; these zinc fingers are minimally required for stable DNA binding under relatively non-stringent conditions of ionic strength. Zinc finger 5 is a critical and context-dependent determinant of stable binding and represents the extent of the minimal DNA-binding domain under more stringent binding conditions. Zinc fingers 6 and 7, although nonessential, nonetheless further stabilize DNA binding mediated by zinc fingers 1–5. Finally, zinc finger 8 and the C terminus apparently destabilize the maximum potential DNA binding activity inherent in zinc fingers 1–7.

Identification of BRCA1-binding Determinants on ZBRK1—Next, we sought to establish more precisely the molecular determinants on ZBRK1 required for BRCA1 binding. Previously, we mapped the BRCA1-binding domain on ZBRK1 to encompass a broad region extending from zinc finger 5 through the C terminus (5ZFC) (39). To more narrowly define the BRCA1-binding determinants on ZBRK1, we examined the contribution of individual zinc fingers 5–8 as well as sequences within the ZBRK1 C terminus to BRCA1 binding using a yeast two-hybrid interaction assay. To this end, individual substitution and truncation mutations within ZBRK1 5ZFC were translationally fused to the GAL4 transactivation domain and tested for their respective abilities to bind to the ZBRK1-interaction domain on BRCA1 (amino acids 341–748), translationally fused to the GAL4 DNA-binding domain in yeast (Fig. 3). Corresponding β-galactosidase activities identified critical determinants of BRCA1 interaction on ZBRK1 to include the C terminus as well as zinc fingers 7 and 8 (Fig. 3). Deletion of only 9 amino acids from the C terminus significantly compromised BRCA1 binding, indicating that the unique C terminus on ZBRK1 is required in its entirety for efficient interaction with BRCA1 (Fig. 3). This observation sug-

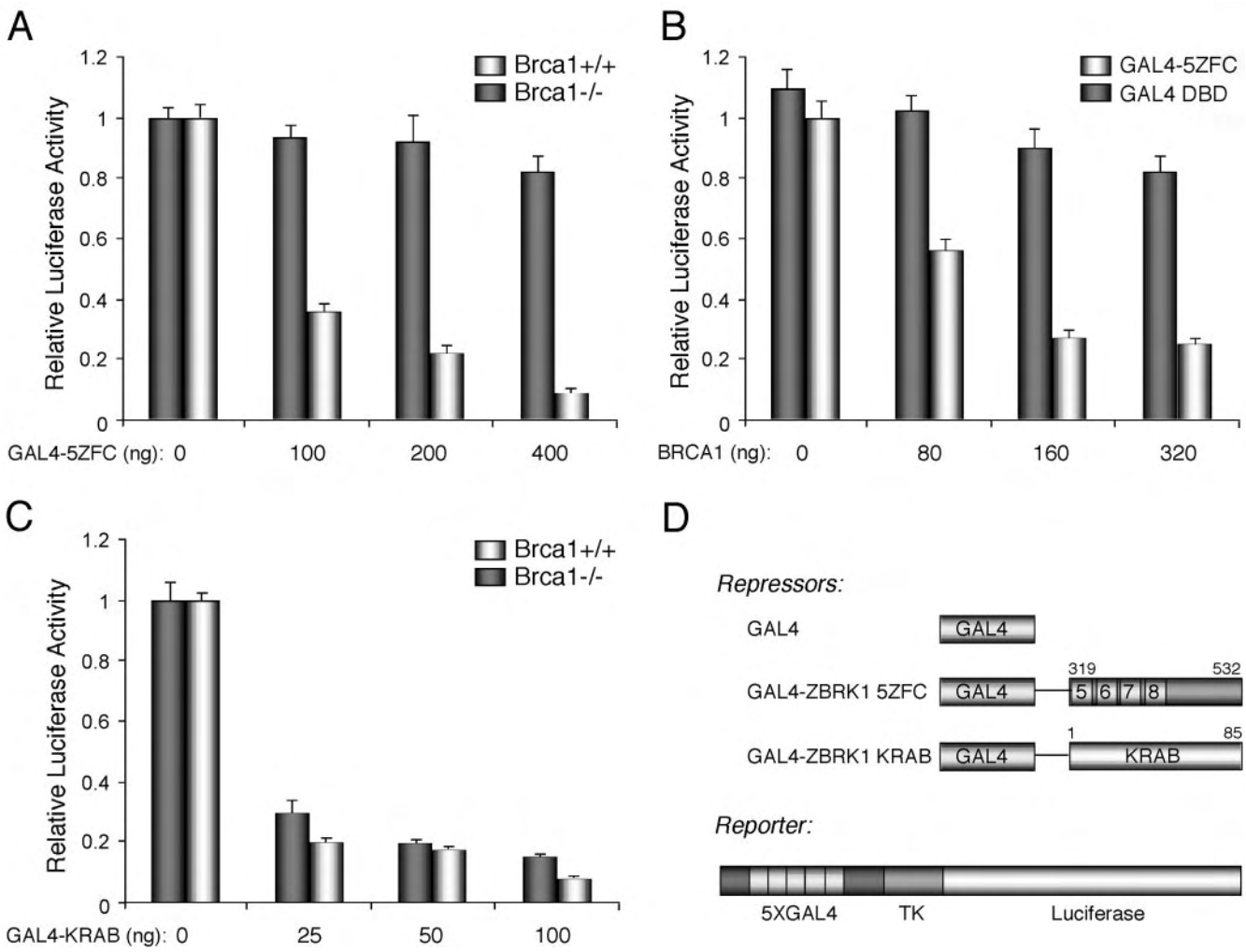

FIG. 5. **The ZBRK1 5ZFC and KRAB repression domains function in a BRCA1-dependent and BRCA1-independent manner, respectively.** *A*, *Brca1*+/+ and *Brca1*−/− MEF cells (39) as indicated were transfected with 100 ng of pG_5TK-LUC without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC. *B*, *Brca1*−/− MEF cells were transfected with 100 ng of pG_5TK-Luc and 20 ng of either pM (expressing the GAL4 DNA-binding domain (*DBD*) alone) or GAL4-ZBRK1 5ZFC, respectively, without or with the indicated nanogram amounts of pCS2+-BRCA1 expressing wild-type human BRCA1. *C*, *Brca1*+/+ and *Brca1*−/− MEF cells as indicated were transfected with 100 ng of pG_5TK-Luc without or with the indicated nanogram amounts of GAL4-ZBRK1 KRAB. *A–C*, relative luciferase activities were calculated as described in the legend to Fig. 4. *D*, schematic representation of the GAL4 DNA-binding domain, the GAL4-ZBRK1 5ZFC and GAL4-ZBRK1 KRAB chimeras, and the pG_5TK-Luc reporter template used in transfection assays.

gests that the overall conformation of the C terminus is likely to be important for BRCA1 interaction. Whereas ZBRK1 zinc fingers 7 and 8 are critical for BRCA1 interaction, zinc fingers 5 and 6 do not appear to contribute to BRCA1 binding (Fig. 3). Taken together, these results indicate that important BRCA1-binding determinants on ZBRK1 include those that also modulate its sequence-specific DNA binding activity in both a positive (zinc finger 7) and negative (zinc finger 8 and the C terminus) manner.

The BRCA1-binding Domain on ZBRK1 Functions as an Autonomous BRCA1-dependent Transcriptional Repression Domain—The fact that BRCA1 contacts ZBRK1 through surfaces that are not essential but nonetheless modulatory with respect to DNA binding suggests several potential mechanisms by which BRCA1 might mediate transcriptional repression by ZBRK1. First, BRCA1 could mediate ZBRK1 repression, at least in part, by modulating its sequence-specific association with DNA. This possibility is currently under investigation. Alternatively, or additionally, BRCA1 could mediate repression by DNA-bound ZBRK1. This possibility is supported by our previous observation that clinically validated missense mutations within the BRCA1 C terminus that do not disrupt its interaction with ZBRK1 nonetheless abrogate its ZBRK1 co-repressor activity (39). To test this possibility directly, we examined whether the BRCA1-binding domain on ZBRK1 could function as a BRCA1-dependent transcriptional repression domain when tethered to a heterologous DNA-binding domain. This approach permitted us to assess the influence of BRCA1 on the repression function of ZBRK1 independently of any effects that it might have on the DNA binding activity of ZBRK1. Accordingly, we initially tested the ability of ZBRK1 5ZFC (extending from zinc finger 5 to the C terminus) to function as an independent repression domain when linked to the GAL4 DNA-binding domain. GAL4-ZBRK1 5ZFC was transiently expressed in U2OS human osteosarcoma cells, and its influence on transcription from a pG_5TK-Luc reporter template bearing five copies of the consensus GAL4 DNA-binding site upstream of the herpes simplex virus (HSV) TK gene promoter was examined. GAL4-ZBRK1 5ZFC conferred greater than 10-fold repression upon reporter gene expression in a dose-dependent manner (Fig. 4*A*). We also confirmed the presence of a potent KRAB repression domain within the ZBRK1 N terminus by examining its ability repress pG_5TK-Luc reporter gene expression when tethered to the GAL4 DNA-binding domain (Fig. 4*B*). Based on quantitative immunoblot analysis of transfected

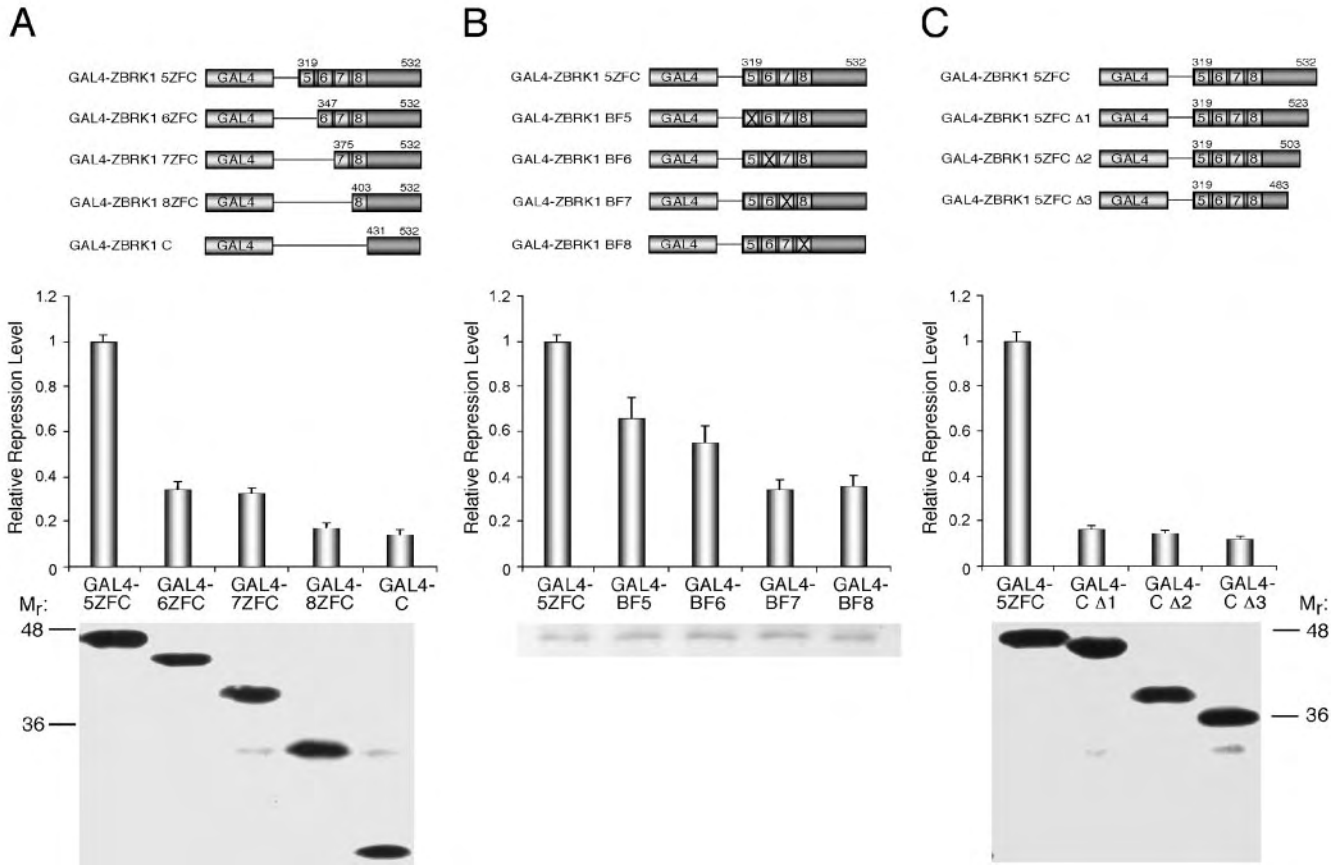

FIG. 6. **Functional analysis of ZBRK1 5ZFC truncation and substitution derivatives.** *A–C*, U2OS cells were transfected with 30 ng of pG_5TK-Luc and the following amounts of GAL4-ZBRK1 5ZFC or its indicated derivatives: *A*, 5ZFC, 400 ng; 6ZFC, 7ZFC, 8ZFC, and C, 200 ng; *B*, 5ZFC or its indicated broken finger derivatives, 200 ng; *C*, 5ZFC, 400 ng; Δ1, 200 ng; Δ2, 400 ng; Δ3, 200 ng. In each panel, the relative repression level represents the relative luciferase activity obtained with a particular GAL4-ZBRK1 5ZFC derivative divided by that obtained with GAL4-ZBRK1 5ZFC. Relative luciferase activities were calculated as described in the legend to Fig. 4. In these experiments, the relative luciferase activity observed with GAL4-ZBRK1 5ZFC (three independent transfections performed in duplicate) was as follows: *A*, 0.10 corresponding to 10-fold repression of reporter template activity; *B*, 0.21 corresponding to an approximate 5-fold repression of reporter template activity; *C*, 0.08 corresponding to 12.5-fold repression of reporter template activity. To confirm that GAL4-ZBRK1 5ZFC and its truncation and substitution derivatives are expressed at roughly equivalent levels, lysates from U2OS cells transfected with GAL4-ZBRK1 5ZFC or its derivatives (the same amounts used in functional analysis and indicated above) were resolved by SDS-10% PAGE and subjected to immunoblot analysis using antibodies specific for the GAL4 DNA-binding domain. Molecular weight marker positions (M_r) are indicated. GAL4-ZBRK1 5ZFC and its derivatives are represented schematically at the *top* of each panel.

cell extracts, the ZBRK1 KRAB domain, on a molar basis, appears to be a stronger transcriptional repression domain than the ZBRK1 BRCA1-binding domain (data not shown). Nonetheless, this result indicates the presence within ZBRK1 of two portable transcriptional repression domains, an N-terminal KRAB domain and a novel C-terminal transcriptional repression domain (5ZFC) that encompasses the BRCA1-binding domain.

To confirm the requirement for BRCA1 in transcriptional repression by ZBRK1 5ZFC, we tested the repression function of GAL4-ZBRK1 5ZFC in *Brca1*+/+ and *Brca1*−/− MEF cells. GAL4-ZBRK1 5ZFC conferred up to 10-fold repression in *Brca1*+/+ MEFs, whereas little or no repression activity was observed in *Brca1*−/− MEFs (Fig. 5*A*). Ectopic expression of BRCA1 in *Brca1*−/− MEFs restored ZBRK1 5ZFC-directed transcriptional repression (Fig. 5*B*), establishing conclusively that BRCA1 mediates repression by DNA-bound ZBRK1 5ZFC. In contrast to ZBRK1 5ZFC, the ZBRK1 KRAB domain repressed transcription equivalently in both *Brca1*+/+ and *Brca1*−/− MEFs (Fig. 5*C*). Thus, the N-terminal KRAB and C-terminal 5ZFC repression domains within ZBRK1 can be distinguished functionally on the basis of their respective requirements for BRCA1.

BRCA1-binding Is Necessary but Not Sufficient for ZBRK1 5ZFC Repression Function—To more narrowly define the boundaries of the BRCA1-dependent 5ZFC repression domain within ZBRK1, we examined a panel of ZBRK1 5ZFC truncation and substitution mutants for their respective repression activities *in vivo*. Relative to the intact 5ZFC domain, deletion or disruption of zinc fingers 5 or 6 individually reduced repression activity by 2–3-fold (Fig. 6, *A* and *B*), whereas individual deletion or disruption of zinc fingers 7 or 8 reduced repression activity by 3–6-fold (Fig. 6, *A* and *B*). These results indicate that zinc fingers 5–8 are all required for the integrity of the 5ZFC repression domain, although zinc fingers 7 and 8 appear to be quantitatively more important. Deletion of only 9 amino acids from the C terminus of ZBRK1 severely compromised the repression function of the 5ZFC domain, indicating that the entire C terminus is likely to be important for 5ZFC repression activity (Fig. 6*C*). Thus, the 5ZFC repression domain extending from ZBRK1 zinc finger 5 through the C terminus appears to constitute an intact repression domain that cannot be further delimited. This analysis also reveals an imperfect correlation between BRCA1 binding and transcriptional repression by the 5ZFC repression domain. Thus, disruption of ZBRK1 zinc fingers 7 or 8 or truncation of the C terminus severely compromised BRCA1 binding (Fig. 3) and transcriptional repression (Fig. 6). By contrast, disruption of zinc fingers 5 or 6, which are

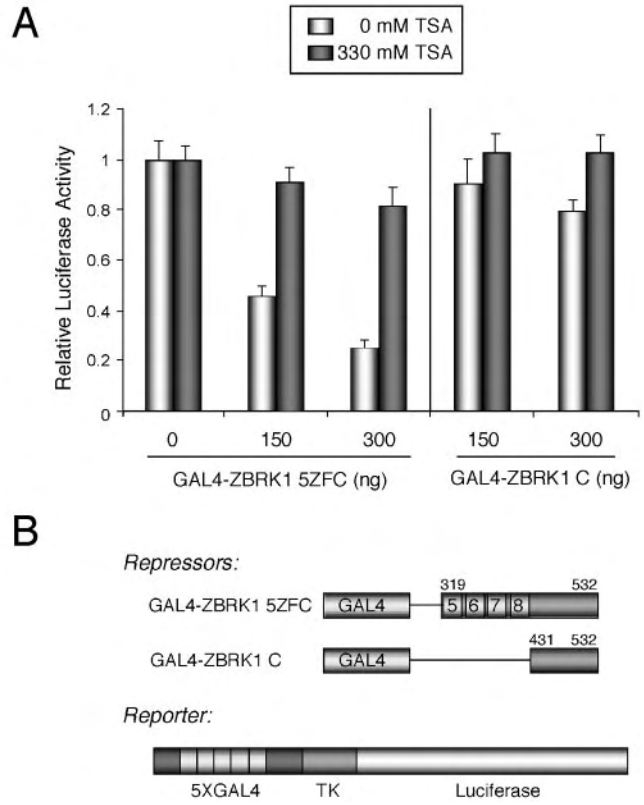

FIG. 7. **ZBRK1 5ZFC-directed repression is reversed by trichostatin A.** *A*, U2OS cells were transfected with 30 ng of pG_5TK-Luc without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC or GAL4-ZBRK1 C. Where indicated, TSA (330 nM) was also included. Relative luciferase activities were calculated as described in the legend to Fig. 4. *B*, schematic representation of the GAL4-ZBRK1 5ZFC and GAL4-ZBRK1 C chimeras and the pG_5TK-Luc reporter template used in transfection assays.

not required for BRCA1 binding (Fig. 3), nonetheless significantly compromised transcriptional repression (Fig. 6, *A* and *B*). On this basis we conclude that BRCA1 binding is necessary but not sufficient for 5ZFC repression function. This suggests that ZBRK1 zinc fingers 5 and 6 may possibly contact an additional co-repressor(s).

The BRCA1-dependent 5ZFC Repression Domain Is Histone Deacetylase-dependent and Promoter-specific—Previously, BRCA1 has been shown to interact through its C-terminal BRCT repeats with histone deacetylases (HDACs) 1 and 2 (57). HDACs remove acetyl groups from lysine residues on histone tails and thus promote the formation of transcriptionally repressive chromatin. To determine the contribution of HDAC activity to repression mediated by ZBRK1 5ZFC, we tested the effect of the selective HDAC inhibitor trichostatin A (TSA) on repression mediated by GAL4-ZBRK1 5ZFC. TSA largely reversed GAL4-ZBRK1 5ZFC repression in U2OS cells, implicating HDAC activity in this process (Fig. 7).

Like the ZBRK1 5ZFC repression domain, KRAB repression domains function through HDACs as well as through histone methyltransferases and heterochromatin proteins (41–47). This prompted us to comparatively examine the promoter specificities of the ZBRK1 5ZFC and KRAB repression domains. The ZBRK1 N-terminal KRAB domain repressed transcription potently from each of three different RNA polymerase II promoters tested: the SV40 major late, the HSV TK, and the human small nuclear ribonucleoprotein N (SNRPN) promoters (Fig. 8*B*). By contrast, ZBRK1 5ZF5C repressed transcription potently from the HSV TK promoter, moderately from the SV40 promoter, and not at all from the SNRPN promoter (Fig. 8A). These results indicate that the ZBRK1 KRAB and 5ZFC repression domains can be distinguished functionally not only by their requirement for BRCA1 but also on the basis of their promoter specificities; whereas the ZBRK1 KRAB repression domain exhibits broad promoter selectivity, the BRCA1-dependent 5ZFC repression domain exhibits a more restricted promoter bias.

DISCUSSION

A central question regarding the role of BRCA1 in transcription control concerns the means by which it mediates gene-specific regulation in the absence of sequence-specific DNA binding activity. In part, this question has been answered by the identification of a growing number of sequence-specific DNA-binding transcription factors with which BRCA1 physically and functionally interacts. In this regard, our previous identification of ZBRK1 as a BRCA1-dependent transcriptional repressor provided a molecular basis to link BRCA1 directly to the regulation of *GADD45a* gene expression (39). Other work has rendered it clear that the BRCA1 regulation of *GADD45a* gene transcription is likely to be complex and mediated not only through ZBRK1 but other trans-acting factors, including OCT1 and NF-YA (21, 32, 39). Presently, however, little is known regarding the mechanism(s) by which BRCA1 mediates sequence-specific transcriptional control through the various transcription factors with which it interacts. Here we have investigated the functional interaction between ZBRK1 and BRCA1 in an effort to understand the role of BRCA1 in sequence-specific transcriptional repression.

Our studies suggest that BRCA1 mediates ZBRK1 repression, at least in part, through its targeted recruitment to a novel C-terminal repression domain (5ZFC) within ZBRK1. Structurally, this repression domain comprises the last four zinc fingers and the unique C-terminal extension of ZBRK1. The identification of 5ZFC as a discrete functional domain was revealed by its ability to repress transcription when tethered to a heterologous DNA-binding domain (Fig. 4) and its functional resistance to truncation or substitution mutagenesis (Fig. 6). Importantly, we demonstrated that 5ZFC repression function is dependent upon BRCA1; genetic ablation of *Brca1* or disruption of BRCA1-binding determinants on 5ZFC similarly abrogates the repression function of this domain (Figs. 5 and 6). The functional contribution of this domain to BRCA1-dependent ZBRK1 repression is reflected by our previous observation that deletion of the ZBRK1 C terminus abrogates ZBRK1 repression through natural ZBRK1-response elements (39). However, whereas BRCA1 binding is necessary, our studies here suggest that it is not sufficient for 5ZFC-directed repression. First, BRCA1 binding and repression determinants within this domain can be separated, suggesting a possible functional requirement for a co-repressor(s) in addition to BRCA1 (Figs. 3 and 6). Second, 5ZFC-directed repression is HDAC-dependent (Fig. 7). Thus, we propose that the ZBRK1 5ZFC repression domain recruits BRCA1 as part of a higher order repression complex that minimally includes an associated HDAC activity. Targeted attempts to identify the functionally relevant BRCA1-associated co-repressor activities are currently underway.

Our work further reveals unique insight into the structural and functional organization of ZBRK1, a member of the KRAB-ZFP family. The ~220 members of this family make up a significant proportion of the transcription factor complement of the human proteome and are believed to occupy important regulatory roles in development, differentiation, and transformation (41, 58–63). Despite their potential biological significance, our current understanding of the mechanisms through

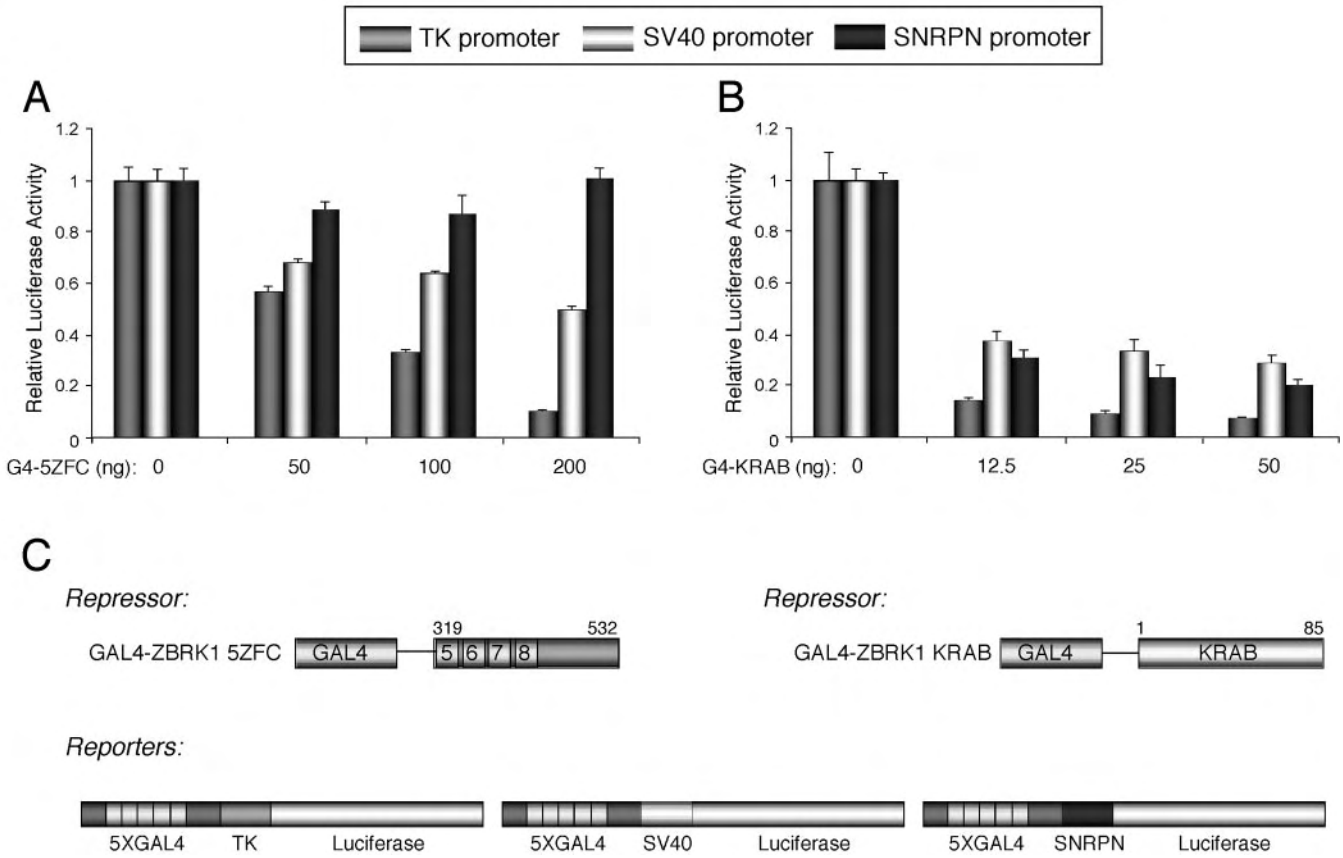

FIG. 8. **The ZBRK1 KRAB and 5ZFC repression domains exhibit unique promoter specificities.** *A* and *B*, U2OS cells were transfected with 30 ng of pG_5TK-Luc, 15 ng pG_5SV40-Luc, or 90 ng pG_5SNRPN-Luc as indicated without or with the indicated nanogram amounts of GAL4-ZBRK1 5ZFC (*A*) or GAL4-ZBRK1 KRAB (*B*). Relative luciferase activities were calculated as described in the legend to Fig. 4. *C*, schematic representation of the GAL4-ZBRK1 KRAB and GAL4-ZBRK1 5ZFC chimeras, and the pG_5TK-Luc, pG_5SV40-Luc, and pG_5SNRPN-Luc reporter templates used in transfections.

which individual members of this protein family function is still rather limited. Thus, although considerable mechanistic insight into the repression function of the KRAB domain has been revealed in recent years (41–47), comparatively little is known regarding the role of KRAB domain-associated zinc fingers in transcriptional repression apart from their presumed role in sequence-specific DNA binding. In part, this gap in knowledge derives from the limited availability of KRAB-ZFP target sequences with which structure-function analyses may be carried out. In the case of several KRAB-ZFPs whose corresponding binding site sequences have been identified, an additional function(s) for individual zinc fingers beyond DNA binding seems implicit. For example, based on the observation that one C_2H_2 zinc finger can bind to ~3 bp of DNA (52–54), the established target sequence lengths of 5 and 27 bp, respectively, for the 8- and 10-fingered ZNF202 and KS1 proteins are incompatible with DNA contact mediated by every zinc finger (61, 63). Our previous derivation of a consensus binding sequence for ZBRK1 has permitted us here to dissect a long array KRAB-ZFP and examine the contribution of individual zinc fingers to both sequence-specific DNA-binding and transcriptional repression. Our studies reveal the ZBRK1 zinc fingers to be multifunctional in nature, with dedicated roles in binding DNA, BRCA1, or both.

First, zinc fingers 1–4 are essential for DNA binding activity and compose the minimal DNA-binding domain under moderate conditions of ionic strength (Fig. 1). Zinc finger 5 appears to be a critical and context-dependent DNA-binding zinc finger; this finger represents the extent of the minimal DNA-binding domain under more stringent conditions (Fig. 1). Zinc fingers 6 and 7 are not essential for DNA binding but nonetheless enhance the stability of DNA binding (Figs. 1 and 2). Finally, zinc finger 8 (along with the C terminus) is dispensable for, and may possibly destabilize, DNA binding mediated by zinc fingers 1–7 (Fig. 1). Taken together, these findings reveal the ZBRK1 zinc fingers to compose at least two functional classes: those that make minimal essential contacts with DNA (fingers 1–4) and those that modulate the stability of these contacts (fingers 5–8). Importantly, zinc fingers 5–8 that modulate ZBRK1 DNA binding activity also represent critical determinants of repression by DNA-bound ZBRK1 through association with co-repressors, including BRCA1. These findings thus extend the established role of KRAB-zinc fingers to include protein-protein interactions critical for transcriptional repression, and also identify within ZBRK1 dual specificity zinc fingers with twin roles in DNA-binding and transcriptional repression.

Our work advances the understanding of DNA recognition by KRAB-ZFPs in several respects. First, we provide further empirical evidence to support predictive models for C_2H_2 zinc finger-DNA recognition. Structural studies of 3- and 5-fingered proteins in complex with DNA have indicated that individual zinc fingers bind to ~3 bp of DNA (52–54). Based on this model, five of the eight ZBRK1 zinc fingers would be predicted to bind to its 15-bp recognition sequence. In fact, DNA-binding analyses revealed that under mild conditions of ionic strength, the first four ZBRK1 zinc fingers are sufficient to confer stable binding to its consensus sequence. However, more stringent conditions unmasked a requirement for the fifth finger, consistent with the aforementioned structural models. Second, our identification of ZBRK1 zinc finger 5 as a critical and context-

dependent DNA-binding determinant could clarify recent issues concerning selectivity among KRAB-ZFPs that recognize overlapping DNA-binding site sequences. In this regard, a four-fingered KRAB-ZFP called SZF1 was recently shown to recognize a DNA-binding site in common with ZBRK1 (64). The observation that SZF1 and ZBRK1 exhibit overlapping DNA-binding specificities *in vitro* raises the possibility that these proteins might compete for a common binding site(s) *in vivo* (64). This, in turn, could have significant implications for the biological regulation of target gene transcription by each of these proteins. However, as we show here, ZBRK1 zinc finger 5 is a critical DNA-binding determinant under more stringent conditions of increased ionic strength and also increased nonspecific competitor concentrations *in vitro* (Fig. 1 and data not shown). Because these conditions are more likely to approximate those of the cellular milieu, in which target site location must be achieved in the presence of a vast excess of like and unlike DNA sequences, ZBRK1 zinc finger 5 could represent a critical determinant of target site selection *in vivo*. Beyond zinc finger 5, zinc fingers 6 and 7 through enhanced affinity and/or protein-protein interactions could further influence ZBRK1 target site selectivity.

Our identification within ZBRK1 of a C-terminal BRCA1-dependent repression domain in addition to the N-terminal KRAB domain represents the first demonstration of a KRAB-ZFP harboring two independent repression domains. More importantly, the presence of two inherent repression domains could have important implications for gene-specific transcription control by ZBRK1. As we show here, the KRAB and C-terminal repression domains within ZBRK1 can be distinguished functionally on the basis of their respective requirements for BRCA1; the C-terminal repression domain is BRCA1-dependent, whereas the KRAB domain is not. This functional distinction may in part underlie the unique promoter specificities of the two repression domains. Whereas the KRAB repression domain exhibits broad promoter specificity, the BRCA1-dependent repression domain exhibits a more restricted promoter bias. Thus, the relative contribution of the BRCA1-dependent repression domain to overall ZBRK1 repression may vary among different ZBRK1 target promoters, effectively expanding the regulatory potential available at ZBRK1 target genes. It will be of interest in future studies to determine whether and how these discrete repression domains function synergistically to confer ZBRK1 repression.

Finally, although our work suggests that BRCA1 mediates repression by DNA-bound ZBRK1, we cannot exclude the additional possibility that BRCA1 also mediates ZBRK1 repression, at least in part, by modulating it sequence-specific DNA binding activity. Our observation that the BRCA1-binding surface on ZBRK1 includes zinc fingers that modulate its DNA binding activity in both a positive (zinc finger 7) and negative (zinc finger 8) manner is consistent with this possibility, and studies are currently underway to address this important issue. Nonetheless the studies presented here shed new light on the functional organization of ZBRK1 as a model KRAB-ZFP and further define the role of BRCA1 in sequence-specific transcription control.

Acknowledgments—We thank our laboratory colleagues for advice and discussion and P. Renee Yew for insight and comments. We thank Drs. Y. Tony Ip (University of Massachusetts Medical School, Worcester, MA) and Paul A. Wade (Emory University School of Medicine, Atlanta, GA) for reporter plasmids.

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Appendix 6

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 53, Issue of December 31, pp. 55153–55160, 2004
Printed in U.S.A.

Tetrameric Oligomerization Mediates Transcriptional Repression by the BRCA1-dependent Kruppel-associated Box-Zinc Finger Protein ZBRK1*

Received for publication, September 22, 2004, and in revised form, October 5, 2004
Published, JBC Papers in Press, October 20, 2004, DOI 10.1074/jbc.M410926200

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The Kruppel-associated box (KRAB)-zinc finger protein ZBRK1 has been implicated in the transcriptional regulation of DNA damage-response genes that function in cell growth control and survival. Recently, we described a novel BRCA1-dependent C-terminal transcriptional repression domain (CTRD) within ZBRK1, the mode of repression of which is functionally distinguishable from that of the N-terminal KRAB repression domain within ZBRK1. The identification of BRCA1 binding-competent but repression-defective CTRD mutants further revealed that BRCA1 binding is necessary, but not sufficient, for ZBRK1 CTRD function. During an unbiased search for possible co-regulators of the CTRD, we identified ZBRK1 itself, suggesting that ZBRK1 can oligomerize through its CTRD. Herein we explore the physical and functional requirements for ZBRK1 oligomerization in ZBRK1-directed transcriptional repression. Protein interaction analyses confirmed that ZBRK1 can homo-oligomerize both *in vitro* and *in vivo* and further mapped the ZBRK1 oligomerization domain to the CTRD C terminus. Biochemical analyses, including protein cross-linking and gel filtration chromatography, revealed that ZBRK1 homo-oligomers exist as tetramers in solution. Functionally, ZBRK1 oligomerization facilitates ZBRK1-directed transcriptional repression through ZBRK1 response elements; requirements for oligomerization-dependent repression include the ZBRK1 CTRD and KRAB repression domains but not the DNA binding activity of ZBRK1. These observations suggest that higher order oligomers of ZBRK1 may assemble on target ZBRK1 response elements through both protein-DNA and CTRD-dependent protein-protein interactions. These findings thus reveal an unanticipated dual function for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression and further establish the CTRD as a novel protein interaction surface responsible for directing homotypic and heterotypic interactions necessary for ZBRK1-directed transcriptional repression.

ZBRK1[1] (zinc finger and BRCA1-interacting protein with a KRAB domain-1) is a member of the Kruppel-associated box-zinc finger protein (KRAB-ZFP) family of transcriptional repressors (1, 2). The ~300 members of this family comprise a significant proportion of the transcription factor complement of the human proteome and are believed to occupy important regulatory roles in development, differentiation, and transformation (3–9). In addition to its identification as the product of a gene up-regulated in senescent fibroblasts, ZBRK1 was independently identified as a BRCA1-dependent transcriptional repressor of the gene encoding GADD45a, a functionally important DNA damage-response effector that functions in G_2/M cell cycle checkpoint control and the maintenance of genomic stability (2, 10, 11). Previous functional analyses revealed that ZBRK1 represses *GADD45a* gene transcription through an intron 3 DNA-binding site in a manner dependent upon direct interaction with BRCA1 and further suggested a model in which ZBRK1 and BRCA1 function coordinately to repress *GADD45a* gene transcription in the absence of genotoxic stress (2). Evidence to support this model derives from the recent observation that DNA damage-induced ubiquitin-mediated proteolysis of ZBRK1 leads to de-repression of *GADD45a* (12). Although the DNA damage-induced degradation of ZBRK1 is a BRCA1-independent phenomenon, familial breast cancer-derived mutants of BRCA1 that disrupt its interaction with ZBRK1 nonetheless abrogate its co-repressor activity, suggesting that its ZBRK1 co-repressor function may be important for the tumor suppressor properties of BRCA1 (2, 12).

In addition to *GADD45a*, potential ZBRK1 binding sites have been identified in other DNA damage-response genes that are also regulated by BRCA1, including *p21*, *Bax*, and *GADD153* (2). This observation suggests a potentially broader role for BRCA1 and ZBRK1 in the coordinate transcriptional control of functionally diverse DNA damage response genes. As part of our initial effort to explore the mechanism by which BRCA1 mediates sequence-specific transcriptional repression through ZBRK1, we recently identified and functionally characterized a novel C-terminal transcriptional repression domain (CTRD) within ZBRK1 (13). Structurally, the CTRD comprises the last four zinc fingers and an atypical C-terminal extension within the eight-fingered ZBRK1 protein (13). Functionally, the CTRD represses transcription in a BRCA1-dependent, histone deacetylase-dependent, and promoter-specific manner and is thus distinguishable from the N-terminal KRAB repres-

* This work was supported by United States Army Department of Defense Grant DAMD17-02-1-0584, National Institutes of Health Grant CA098301-01 (to T. G. B.), and United States Army Department of Defense Predoctoral Fellowship DAMD17-03-1-0259 (to W. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ZBRK1, zinc finger and BRCA1-interacting protein with a KRAB domain-1; BF, broken finger; CTRD, C-terminal transcription repression domain; DSS, disuccinimidyl suberate; KRAB, Kruppel-associated box; MBP, maltose-binding protein; TK, thymidine kinase; ZFP, zinc finger protein; ZRE, ZBRK response element.

sion domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity (13).

During the course of our functional dissection of ZBRK1, we identified CTRD mutants that are competent for BRCA1 binding but nonetheless defective for transcriptional repression (13). This observation suggests that BRCA1 binding is necessary but not sufficient for the ZBRK1 CTRD repression function and implies a role for additional co-regulators of the CTRD. During an unbiased search for CTRD-interacting proteins and, therefore, potential CTRD co-regulators, we unexpectedly identified ZBRK1 itself, suggesting that ZBRK1 can homo-oligomerize through its CTRD. Herein, we explore the physical and functional requirements for ZBRK1 oligomerization in ZBRK1-directed repression. Our results reveal the ZBRK1 CTRD to be a novel interaction surface responsible for directing both homotypic and the heterotypic interactions essential for ZBRK1 repression and further suggest an unanticipated dual role for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression. We discuss the implications of these findings for BRCA1-dependent ZBRK1 repression.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—Plasmid pMAL-C2-TEV ZBRK1 ΔK for expressing maltose-binding protein (MBP)-ZBRK1 ΔK in *Escherichia coli* has been described previously (13). pCS2+-FLAG-ZBRK1 for *in vitro* transcription/translation and mammalian expression of FLAG epitope-tagged ZBRK1 was constructed by subcloning a BamHI-HindIII fragment carrying sequences encoding an amino-terminally FLAG-tagged ZBRK1 from pFastbac-FLAG-ZBRK1 into pCS2+ (14). pCS2+-FLAG-ZBRK1 broken finger (BF) and KRAB domain (D12A,V13A) mutants were generated by site-directed mutagenesis using the QuikChange II site-directed mutagenesis kit following the manufacturer's recommendations (Stratagene, La Jolla, CA). Each broken finger mutant bears a histidine (CAT codon) to asparagine (AAT codon) substitution mutation at the first of the two conserved histidine residues within the targeted C_2H_2 zinc finger. The D12A,V13A KRAB domain double mutation bears an aspartic acid (GAT codon) to alanine (GGT) substitution and a valine (GTG codon) to alanine (GGG codon) substitution at amino acid position numbers 12 and 13, respectively, of the 532-amino acid ZBRK1 protein. pCS2+-FLAG-ZBRK1 carboxyl-terminal truncation mutants (ΔC1, ΔC2, ΔC3) were generated by PCR-based subcloning. In brief, a PstI-XbaI fragment encompassing the carboxyl terminus of ZBRK1 in pCS2+-FLAG-ZBRK1 was replaced with PCR-amplified fragments carrying corresponding carboxyl-terminal truncations. Individual pCS2+-FLAG-ZBRK1 carboxyl-terminal deletion derivatives encode the following ZBRK1 amino acids: ΔC1, amino acids 1–523; ΔC2, amino acids1–503; and ΔC3, amino acids 1–483. Plasmid pCS2+-T7-ZBRK1 for *in vitro* transcription/translation and mammalian expression of T7 epitope-tagged ZBRK1 was generated by replacing a BamHI-Eco47-III fragment from pCS2+-FLAG-ZBRK1 with a PCR-amplified BamHI-Eco47-III fragment carrying sequences encoding a T7 epitope tag in-frame with the ZBRK1 amino terminus.

Reporter plasmid pZRE_4-TK-Luc was constructed by replacing a HindIII-BglII fragment including 5XGAL4 DNA-binding sites and the thymidine kinase (TK) promoter from pG_5TK-Luc (13) with a HindIII-BglII fragment from pBL-CAT E (2), encompassing 4× ZBRK1 consensus DNA-binding sites and the TK promoter. Plasmid pM-ZBRK1 CTRD (previously named pM-ZBRK1 5ZFC) for mammalian expression of GAL4-CTRD and reporter plasmid pG_5TK-Luc have both been described previously (13).

Cell Culture, Transfections, and Reporter Assays—U2OS human osteosarcoma cells were cultured as described (13). For transient reporter assays, 2×10^5 cells were seeded per well of a 6-well plate 24 h before transfection. When 70% confluent, the cells were transfected with FuGENE 6 (Roche Diagnostics) following the manufacturer's instructions. The amounts of expression and reporter plasmids utilized in each experiment are indicated in the legends to Figs. 4–6. A total of 1or 2 μg, respectively, of DNA per well was transfected when pG_5TK-Luc or pZRE_4-Luc were used as reporter templates. Each transfection also included an internal control plasmid, pCH110 (15), expressing β-galactosidase under control of the SV40 promoter. Forty-eight hours post-transfection, cells were harvested and lysed in 250 μl/well reporter lysis buffer (Promega, Madison, WI). Transfected cell lysates (20 μl) were analyzed for luciferase activity using the luciferase assay system (Promega) and for β-galactosidase activity using the Galacto-Light Plus chemiluminescent reporter assay (BD Biosciences). Each transfection was performed a minimum of three times in duplicate.

Protein Expression and Purification—MBP-ZBRK1 ΔK, MBP-ZBRK1 CTRD, and MBP proteins were each expressed in and purified from *E. coli* strain BL21 Star (DE3) pLysS (Invitrogen). The procedures for protein induction, extraction, and purification of MBP-ZBRK1 ΔK have been described previously (13). For MBP-ZBRK1 CTRD and MBP, cells were grown at 37 °C to an A_{600} of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration 0.3 mM, and the cells were transferred to 25 °C for another 3.5 h. Cells were pelleted, washed once with phosphate-buffered saline, and then resuspended in MBP binding buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 500 mM NaCl, and 10 mM β-mercaptoethanol) supplemented with protease inhibitors (0.4 μg/ml aprotinin, 0.5 μg/ml chymostatin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine-HCl). Resuspended cells were frozen and thawed once followed by sonication (three times for 1 min each on ice) and clarification by centrifugation at 30,000 × *g* for 30 min. MBP-ZBRK1 CTRD and MBP proteins were purified from clarified lysates by affinity chromatography on amylose resin (New England Biolabs, Beverly, MA). Briefly, clarified lysates were incubated with amylose resin in batch at for 1 h at 4 °C, washed with MBP binding buffer, and eluted with MBP binding buffer containing 0.5% maltose in column format.

MBP Binding Assays—MBP-ZBRK1 ΔK was purified from clarified bacterial cell extract (150 μl) by incubation with amylose resin (15 μl) in batch for 30 min at 25 °C, followed by washing two times for 5 min each at 25 °C in MBP binding buffer supplemented with protease inhibitors and three times for 5 min each at 25 °C in lysis 300 buffer (50 mM Tris-HCl, 300 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, and 10 mM β-mercaptoethanol) supplemented with protease inhibitors. FLAG-ZBRK1 or its mutant derivatives were labeled with [^{35}S]methionine (TNT SP6 quick coupled transcription/translation system; Promega) and incubated with amylose-immobilized MBP-ZBRK1 ΔK in lysis 300 buffer for 1 h at 4 °C. Binding reactions were washed with Lysis 300 buffer four times for 10 min each at 4 °C and subsequently boiled in 30 μl of 1.5× Laemmli sample buffer. Eluates were resolved by SDS-PAGE (10% gel) and visualized by PhosphorImager analysis (Amersham Biosciences).

Co-immunoprecipitation Analyses—U2OS cells at 70% confluency in 10-cm dishes were transfected with 12 μg of DNA using the following expression plasmids (with amounts in parentheses): pCS2+-T7-ZBRK1 (6 μg) and pCS2+FLAG-ZBRK1 (6 μg); pCS2+-T7-ZBRK1 (6 μg) and pCS2+FLAG-ZBRK1 BF mutants (6 μg); pCS2+-T7-ZBRK1 (6 μg) and pCS2+-FLAG-ZBRK1 ΔC1 (3 μg), pCS2+-FLAG-ZBRK1 ΔC2 (4.5 μg), or pCS2+-FLAG-ZBRK1 ΔC3 (2.25 μg) (the total amount of DNA was fixed by supplementing these transfections with pCS2+). Thirty-six hours post-transfection, 2.5×10^6 transfected U2OS cells were harvested, washed in phosphate-buffered saline, and lysed in 300 μl of lysis 150T buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40) by sonication at four intervals of 7 s each using a Branson 450 sonifier (Branson Ultrasonics) at the lowest setting. The lysate was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. Clarified lysates were subjected to co-immunoprecipitation with either 1 μg of anti-T7 antibody (Novagen, Madison, WI) or 5 μg of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) for 4 h at 4 °C. Immunocomplexes were precipitated with 20 μl of protein A-Sepharose (Amersham Biosciences) (for anti-T7 antibody immunoprecipitation) or protein G-Sepharose (Roche Diagnostics) (for anti-FLAG antibody immunoprecipitation) for 2 h at 4 °C. Immunoprecipitates were pelleted and washed with lysis 150T buffer at least four times for 10 min each at 4 °C. Immunoprecipitated proteins were eluted by boiling in 2× Laemmli sample buffer for 3 min, and eluates were subsequently resolved by SDS-PAGE (10% gel) and processed for immunoblot analysis.

Chemical Cross-linking Assays—U2OS cells at 70% confluency on 10-cm plates were transfected with either 12 μg of pCS2+-FLAG-ZBRK1 or 6 μg of pCS2+-FLAG-ZBRK1 ΔC1 in combination with 6 μg of pCS2+. Thirty-six hours post-transfection, cells were harvested and lysed in 300 μl of lysis 150H buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40) by sonication at four intervals of 7 s each using a Branson 450 sonifier (Branson Ultrasonics) at the lowest setting. The lysate was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. Clarified lysates (36 μl) were subjected to chemical cross-linking by the addition of disuccinimidyl suberate (DSS) (25 mM) to a final concentration of 2.25 mM. Cross-linking reactions were performed at 25 °C and terminated by the addition of 2× Laemmli sample buffer followed by boiling for 3 min (16). Cross-linked products were resolved by SDS-PAGE (10% gel) and processed for immunoblot

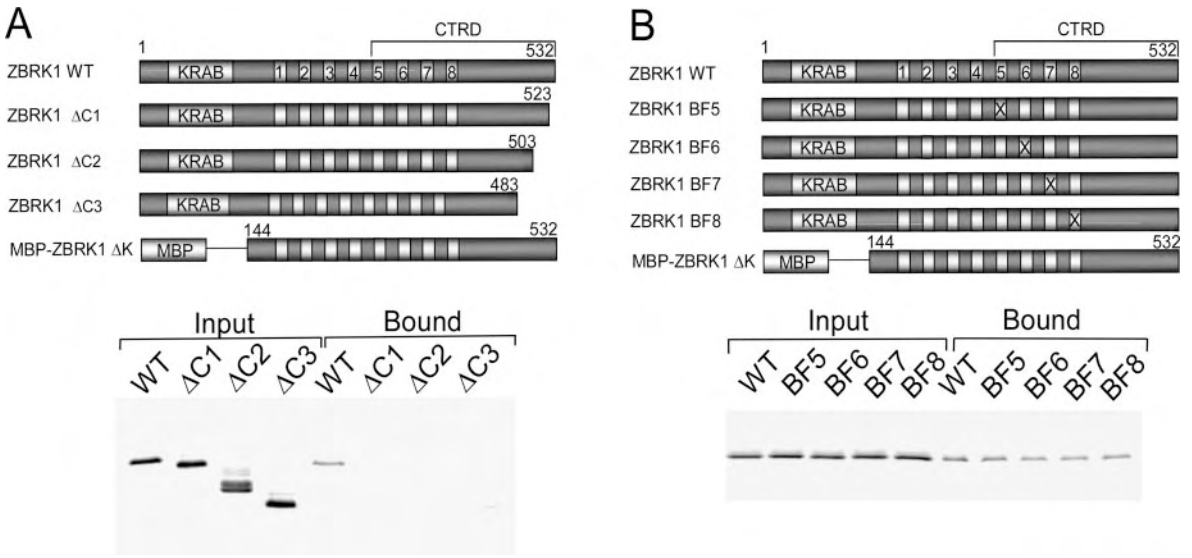

FIG. 1. **ZBRK1 homo-oligomerizes *in vitro* through its CTRD C terminus.** *A* and *B*, *top*, schematic representation of FLAG-tagged wild-type (*WT*) ZBRK1, ZBRK1 carboxyl-terminal deletion (ΔC) and broken finger (*BF*) derivatives, and MBP-ZBRK1 ΔK (the KRAB and CTRD repression domains as well as numbered zinc fingers are indicated). *Bottom*, purified recombinant MBP-ZBRK1 ΔK was immobilized on amylose resin and incubated with *in vitro* translated ^{35}S-labeled wild-type (*WT*) ZBRK1 (*A* and *B*) or its indicated ΔC (*A*) or BF (*B*) derivatives. Following extensive washing, bound proteins were eluted in Laemmli sample buffer, resolved by SDS-PAGE (10% gel), and visualized by PhosphorImager analysis. Input represents 5% of the total *in vitro* translated protein used in binding reactions.

analysis using anti-FLAG M2 monoclonal antibody (Sigma-Aldrich).

Purified MBP-ZBRK1 CTRD and MBP proteins were each subjected to cross-linking by the addition of DSS (25 mM) to a final concentration of 0.75 mM. Cross-linking reactions were performed at 25 °C and terminated by the addition of 2× Laemmli sample buffer followed by boiling for 3 min. Cross-linked products were resolved by SDS-PAGE (10% gel) followed by silver staining.

Gel Filtration Chromatography—U2OS cells at 70% confluency on 10-cm plates were transfected with either 12 μg of pCS2+-FLAG-ZBRK1 or 6 μg of pCS2+-FLAG-ZBRK1 ΔC1 in combination with 6 μg of pCS2+. Thirty-six hours post-transfection, cells were harvested and lysed in 150 μl of lysis 150T buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 25 mM pentaethylene glycol monooctyl ether (Sigma-Aldrich)) by sonication at four intervals of 7 s each using a Branson 450 sonifier (Branson Ultrasonics) at the lowest setting. The lysate was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. Clarified lysates were concentrated 5-fold by centrifugation at 10,000 rpm using a Nanosep 30K omega filtration unit (Pall Corporation, East Hills, NY). Gel filtration chromatography was performed on an Amersham Biosciences SMART System using a Superdex 200 PC 3.2/30 column (Amersham Biosciences) and a flow rate of 40 μl/min. Column fractions (45 μl) were resolved by SDS-PAGE (10% gel) and visualized by immunoblot analysis using an anti-FLAG M2 monoclonal antibody (Sigma-Aldrich).

RESULTS

ZBRK1 Homo-oligomerizes in Vitro—To identify novel interaction partners and potential co-regulators of the ZBRK1 CTRD, we employed two independent experimental approaches. First, we used the CTRD (amino acids 319–532 of ZBRK1) as a bait protein to screen a human fetal brain cDNA library by a yeast-two hybrid assay. From among ~1 × 10^6 independent clones screened, we identified ZBRK1 itself as a CTRD interaction partner (data not shown). Second, we expressed a ZBRK1 KRAB domain deletion derivative (ZBRK1 ΔK; amino acids 144–532 of ZBRK1) as an MBP chimera in *E. coli*, immobilized the fusion protein on amylose resin, and probed HeLa cell nuclear extract for interacting proteins using an MBP pull-down assay. Identification of ZBRK1 ΔK-interacting proteins by matrix-assisted laser desorption ionization time-of-flight-based peptide mass fingerprinting revealed ZBRK1 itself to be among the interaction partners thus captured (data not shown). Coupled with the identification of ZBRK1 as a CTRD-interacting protein by a yeast two-hybrid assay, this observation suggested the possibility that ZBRK1 might homo-oligomerize through a direct interaction involving its CTRD.

To confirm that ZBRK1 can bind directly to itself and to map the relevant interaction surface required for this interaction, we tested the ability of *in vitro* translated wild-type ZBRK1 and ZBRK1 CTRD mutant derivatives for their respective abilities to bind to MBP-ZBRK1 ΔK in an MBP pull-down assay (Fig. 1). As reported previously, deletion of the KRAB domain permits the expression and purification of an otherwise insoluble full-length recombinant ZBRK1 protein (13). MBP-ZBRK1 ΔK bound full-length ZBRK1 efficiently, thus confirming that ZBRK1 can directly self-associate *in vitro* (Fig. 1, *A* and *B*). Next, we examined the structural determinants within the CTRD required for ZBRK1 self-association. Analysis of C-terminal ZBRK1 truncation derivatives revealed that the deletion of only nine amino acids from the CTRD C terminus was sufficient to eliminate ZBRK1 oligomerization (Fig. 1*A*). By contrast, individual disruption of CTRD zinc fingers 5–8 by substitution mutagenesis revealed that each "broken finger" mutant (13) bound MBP-ZBRK1 ΔK comparably to wild-type ZBRK1, thus revealing that the CTRD zinc fingers are individually dispensable for ZBRK1 oligomerization (Fig. 1*B*). Taken together, these results demonstrate that ZBRK1 can homo-oligomerize *in vitro* through a direct interaction involving its CTRD C terminus.

ZBRK1 Homo-oligomerizes in Vivo—To determine whether ZBRK1 can homo-oligomerize *in vivo*, we first examined the ability of ZBRK1 derivatives independently tagged with either T7 or FLAG epitopes to reciprocally co-immunoprecipitate one another following their transient over-expression in U2OS human osteosarcoma cells. T7-tagged ZBRK1 could be efficiently precipitated by FLAG-specific antibodies only in the presence of FLAG-tagged ZBRK1 (Fig. 2*A*). Reciprocally, FLAG-tagged ZBRK1 could be efficiently precipitated by T7-specific antibodies only when T7-tagged ZBRK1 was co-expressed (Fig. 2*B*). These results confirm that ZBRK1 can homo-oligomerize *in vivo*. Next, to map the relevant interaction surface(s) required for ZBRK1 oligomerization *in vivo*, we examined a set of FLAG-tagged ZBRK1 CTRD mutant derivatives for their respective

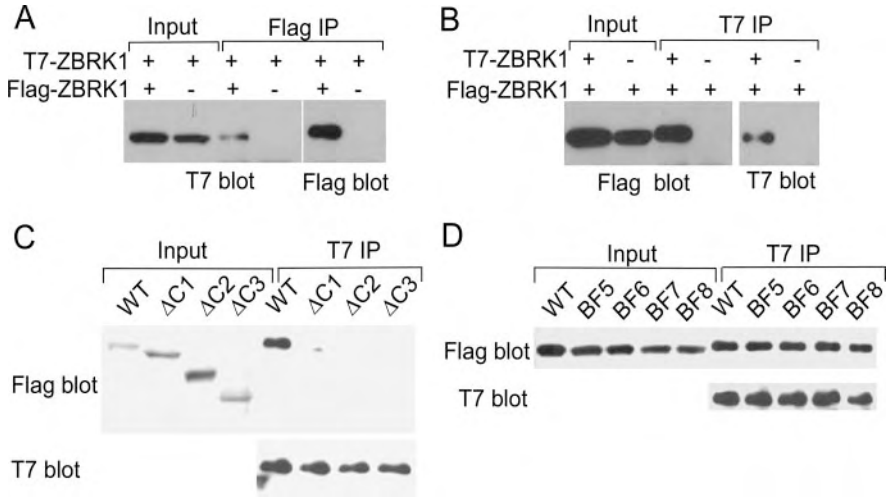

FIG. 2. **ZBRK1 homo-oligomerizes *in vivo* through its CTRD C terminus.** *A* and *B*, T7 and FLAG-tagged wild-type ZBRK1 proteins were ectopically expressed either alone or together in U20S cells, and whole cell extracts from transfected cells were subjected to immunoprecipitation (*IP*) using antibodies specific for either the FLAG (*A*) or T7 (*B*) epitopes. Immunoprecipitates were resolved by SDS-PAGE (10% gel) and processed by immunoblot analysis using both T7 and FLAG epitope-specific antibodies as indicated. Input represents 10% of the total whole cell extract subjected to immunoprecipitation. *C* and *D*, T7-tagged wild-type (*WT*) ZBRK1 was ectopically expressed in U20S cells along with FLAG-tagged WT ZBRK1 or its indicated ΔC (*C*) or BF (*D*) derivatives. Whole cell extracts from transfected cells were subjected to immunoprecipitation (*IP*) using antibodies specific for the T7 epitope. Immunoprecipitates were resolved by SDS-PAGE (10% gel) and processed by immunoblot analysis using both T7- and FLAG epitope-specific antibodies as indicated. Input represents 10% of the total whole cell extract subjected to immunoprecipitation.

abilities to be co-immunoprecipitated along with T7-tagged wild-type ZBRK1 following their transient co-expression in U2OS cells. Consistent with the results of *in vitro* binding analyses, these *in vivo* binding studies revealed the CTRD C terminus to be critical and the CTRD zinc fingers to be dispensable for ZBRK1 self-association. Thus, deletion of only nine amino acids from the ZBRK1 C terminus largely abolished ZBRK1 self-association (Fig. 2*C*), whereas individual ZBRK1 derivatives bearing targeted disruptions of CTRD zinc fingers 5–8 bound to T7-tagged ZBRK1 as efficiently as wild-type ZBRK1 (Fig. 2*D*). Taken together, these results demonstrate that ZBRK1 can homo-oligomerize both *in vitro* and *in vivo* and furthermore delimit the oligomerization interface to the CTRD C terminus.

The ZBRK1 CTRD Mediates Tetrameric Oligomerization—To characterize the oligomeric state of ZBRK1 *in vivo*, we first employed the chemical cross-linker DSS to covalently capture ZBRK1 protein complexes present in extracts of U2OS cells transfected with FLAG-tagged ZBRK1 (16). Cross-linked samples were analyzed by SDS-PAGE and immunoblot analysis using FLAG epitope tag-specific antibodies. In the presence of DSS, a significant proportion of FLAG-tagged ZBRK1 migrated with an apparent molecular mass of ~240 kDa, consistent with the size of a ZBRK1 tetramer (Fig. 3*A*). Importantly, no cross-linked products were observed when an oligomerization-defective ZBRK1 mutant (ΔC1) was over-expressed in U2OS cells, demonstrating that DSS-induced formation of high molecular mass ZBRK1 complexes is strictly dependent upon the intact CTRD C terminus (Fig. 3*A*).

Although the electrophoretic properties of FLAG-tagged ZBRK1 in DSS-cross-linked U2OS cell extracts suggests the formation of tetramers, we could not exclude the possibility that additional cellular proteins were also incorporated into the ZBRK1 complex, particularly because we know that ZBRK1 interacts with other proteins in the cell, including BRCA1 (2, 13). The concentration of ectopically expressed ZBRK1 in these transfection experiments considerably exceeds that of its endogenous counterpart, and these conditions are likely to drive ZBRK1 self-association and mask the influence of endogenous interacting proteins on the size of a cross-linked ZBRK1 complex. Nonetheless, we sought independent confirmation of the stoichiometry of ZBRK1 oligomerization through its CTRD. To this end, we therefore subjected highly purified recombinant MBP-ZBRK1 CTRD to DSS cross-linking. Cross-linked samples were analyzed by SDS-PAGE followed by silver staining to visualize MBP-ZBRK1 CTRD oligomers. In the presence of DSS, a significant proportion of MBP-ZBRK1 CTRD migrated with an apparent molecular mass of ~270 kDa, consistent with the size of an MBP-ZBRK1 CTRD tetramer (Fig. 3*B*). Importantly, no cross-linked products were observed when MBP alone was treated with DSS, indicating that DSS-induced formation of a high molecular mass MBP-ZBRK1 CTRD complex is strictly dependent upon the ZBRK1 CTRD (Fig. 3*B*). Furthermore, immunoblot analysis using ZBRK1-specific antibodies confirmed that the high molecular mass complex observed upon DSS cross-linking of highly purified MBP-ZBRK1 CTRD is derived from MBP-CTRD oligomerization (data not shown).

As an independent approach to determine the stoichiometry of ZBRK1 oligomerization through its CTRD, we examined the respective gel filtration profiles of ectopically expressed wild-type ZBRK1 and its oligomerization-defective mutant (Δ C1) present in extracts of transfected U2OS cells. Immunoblot analysis of column fractions using a FLAG epitope-specific antibody revealed a peak of wild-type ZBRK1 protein in fractions corresponding to an apparent molecular mass of ~240 kDa, most consistent with the size of a ZBRK1 tetramer (Fig. 3*C*). We also noted a minor peak of wild-type ZBRK1 protein in a fraction corresponding to an apparent ZBRK1 monomer of 58 kDa. By contrast, an oligomerization-defective ZBRK1 derivative (Δ C1) peaked exclusively in column fractions corresponding to an apparent monomer of ~57 kDa (Fig. 3*C*). Taken together, these results indicate that ZBRK1 tetramerizes both *in vitro* and *in vivo* in a manner that is strictly dependent upon the integrity of its CTRD C terminus.

ZBRK1 Homo-oligomerization Potentiates ZBRK1-directed Transcriptional Repression—To explore the functional consequence of ZBRK1 homo-oligomerization, we initially exploited our previous observation that the ZBRK1 CTRD can function as an autonomous transcriptional repression domain when tethered to a heterologous DNA-binding domain from the yeast transcription factor GAL4 (13). First, we examined the ability of full-length ZBRK1 to potentiate transcriptional repression

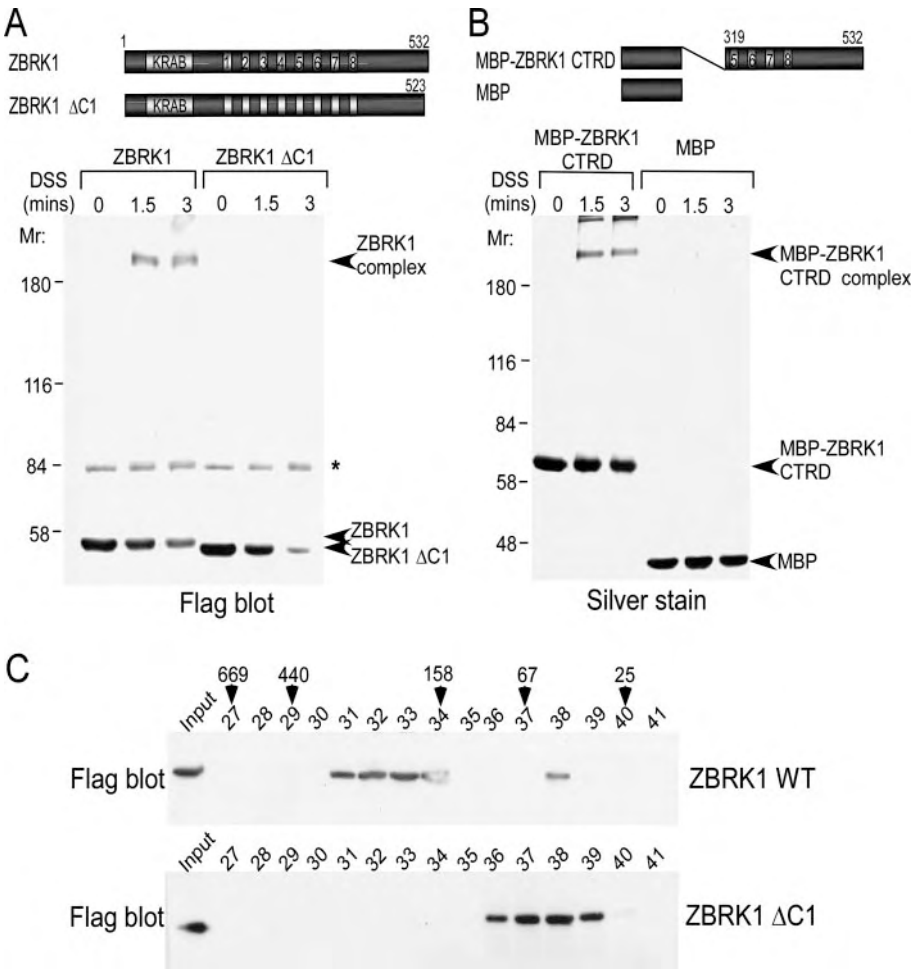

FIG. 3. **ZBRK1 tetramerization requires its CTRD C terminus.** *A*, schematic representation of FLAG-tagged wild-type ZBRK1 and its oligomerization-defective ΔC1 deletion derivative. U2OS cells were transfected with expression vectors for either FLAG-tagged WT ZBRK1 or its ΔC1 deletion derivative as indicated. Whole cell extracts from transfected cells were subjected to cross-linking with 2.25 mM DSS for 0, 1.5, or 3 min as indicated, and cross-linking reactions were terminated with Laemmli sample buffer. Cross-linked proteins were resolved by SDS-PAGE (10% gel) and visualized by immunoblot analysis using FLAG epitope-specific antibodies. *Arrowheads* indicate un-cross-linked monomers of FLAG-tagged ZBRK1 and its ΔC1 deletion derivative as well as cross-linked ZBRK1 complexes. Molecular weight markers (M_r) are indicated. *Asterisk* denotes a cross-reacting cellular protein recognized by the FLAG antibody. *B*, schematic representation of recombinant MBP-ZBRK1 CTRD and MBP. Purified recombinant MBP-ZBRK1 CTRD and MBP were subjected to cross-linking with 0.75 mM DSS for 0, 1.5, or 3 min as indicated, and cross-linking reactions were terminated with Laemmli sample buffer. Cross-linked proteins were resolved by SDS-PAGE (10% gel) and visualized by silver stain analysis. *Arrowheads* indicate un-cross-linked monomers of MBP-ZBRK1 CTRD and MBP as well as cross-linked MBP-ZBRK1 CTRD complexes. Molecular weight markers (M_r) are indicated. *C*, whole cell extracts from U2OS cells transfected with FLAG-tagged wild-type (*WT*) ZBRK1 or an oligomerization-defective mutant derivative (ΔC1) were independently subjected to Superdex 200 gel filtration chromatography. Individual column fractions were resolved by SDS-PAGE (10% gel) and analyzed for the presence of FLAG-tagged ZBRK1 by immunoblot analysis with FLAG epitope-specific antibodies. *Arrowheads* indicate the relative positions of marker protein peaks. Input represents 5% of the total whole cell extract subject to gel filtration chromatography.

directed by GAL4-ZBRK1 CTRD in a transient repression assay using a reporter template bearing multimerized GAL4 DNA-binding sites located upstream of the herpes simplex virus TK promoter in U2OS cells (Fig. 4*A*) (13). Consistent with our previous studies (13), we observed that GAL4-ZBRK1 CTRD repressed transcription from this reporter template at least 10-fold in a dose-dependent manner (data not shown). To examine the influence of wild-type ZBRK1 on repression directed by GAL4-ZBRK1 CTRD, we transfected a sub-optimal nanogram quantity of the GAL4-ZBRK1 CTRD expression vector that supports only ~2.5-fold repression of reporter gene activity. Under these conditions, ectopic expression of wild-type ZBRK1 enhanced this level of repression up to 4-fold in a dose-dependent manner (Fig. 4*B*). Importantly, ZBRK1 had minimal influence on reporter activity in the presence of the GAL4 DNA-binding domain alone, confirming that the repressive effect of ZBRK1 in this assay derives from its direct recruitment to the CTRD and not to DNA (Fig. 4*B*). This result demonstrates for the first time that, in addition to its well documented role as a sequence-specific DNA-binding transcriptional repressor (2, 13), ZBRK1 can also function as a co-repressor independent of its inherent DNA binding activity.

Next, we examined the structural features within ZBRK1 required for its co-repressor function. Relative to wild-type ZBRK1, oligomerization-defective CTRD C-terminal truncation mutants (ΔC1, ΔC2, and ΔC3) were completely defective for ZBRK1 co-repressor activity, implying a strict reliance on oligomerization for ZBRK1 to function as a co-repressor (Fig. 5*A*). Analysis of ZBRK1 derivatives bearing targeted disruptions of CTRD zinc fingers 5–8 (BF 5–8) revealed these mutants to be partially defective for the ZBRK1 co-repressor function (Fig. 5*B*). Because these CTRD zinc finger mutations do not disrupt ZBRK1 oligomerization (see Figs. 1 and 2) but do disrupt CTRD interactions with additional co-repressors including BRCA1 (13), this result suggests that CTRD-mediated recruitment of additional co-repressors also likely contributes to the ability of ZBRK1 to function as a DNA binding-independent co-repressor. Finally, we examined the contribution of the

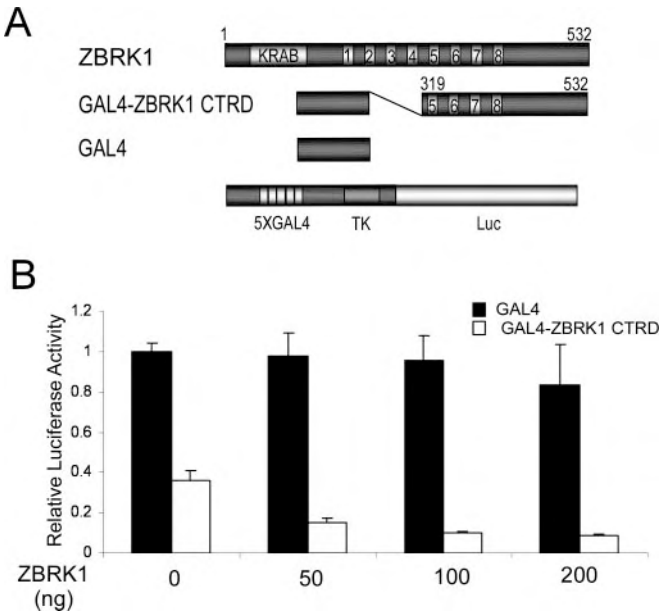

FIG. 4. **ZBRK1 has a DNA binding-independent co-repressor activity.** *A*, schematic representation of FLAG-tagged ZBRK1, GAL4-ZBRK1-CTRD, GAL4 DNA-binding domain alone, and the pG_5TK-luciferase (*5XGAL4 TK Luc*) reporter template used in transfection assays. *B*, U2OS cells were transfected with 100 ng of the pG_5TK-luciferase reporter template and 600 ng of either pM (expressing the GAL4 DNA-binding domain alone) or pM-ZBRK1-CTRD (expressing GAL4-ZBRK1-CTRD) without or with the indicated nanogram amounts of pCS2+-FLAG-ZBRK1 expressing FLAG-tagged wild-type ZBRK1. In this and all subsequent transient reporter assays involving effector plasmid titrations, the total amount of DNA in each transfection was fixed by reciprocal titration of the corresponding backbone expression plasmid. Also, in this and all subsequent transient reporter assays the relative luciferase activity represents the ratio of the luciferase activity obtained in a particular transfection to that obtained in cells transfected with only the reporter and pM expression vectors alone. Luciferase activities were first normalized to β-galactosidase activity obtained by co-transfection of the SV40-β-galactosidase vector (25 ng) as described previously (13). *Error bars* represent the S.D. from the average of at least three independent transfections performed in duplicate.

N-terminal KRAB repression domain within ZBRK1 to its co-repressor activity. Relative to wild-type ZBRK1, a ZBRK1 mutant derivative bearing two substitution mutations (DV → AA) within the KRAB domain previously shown to disrupt KRAB repression function (17) was significantly defective for co-repressor activity (Fig. 5*C*). As this mutation does not affect ZBRK1 oligomerization, this result suggests that KRAB domain-associated co-repressors also contribute to the ability of ZBRK1 to function as a DNA binding-independent co-repressor of the CTRD. Taken together these results reveal that ZBRK1, in a DNA-binding-independent manner, can potentiate the repression function of DNA-bound CTRD through oligomerization and the consequent recruitment of co-repressors through both its KRAB and CTRD repression domains.

We next asked whether ZBRK1 CTRD-mediated oligomerization contributes to ZBRK1-directed transcriptional repression from ZBRK1 response elements (ZREs). To this end, we employed a reciprocal transient repression assay in which we examined the ability of the CTRD to potentiate ZBRK1-directed repression from a reporter template bearing multimerized ZREs (Fig. 6*A*). The fact that the CTRD by itself has no ZRE binding activity permitted us to assess the influence of the CTRD on ZBRK1-directed repression solely by virtue of its ability to oligomerize with DNA-bound ZBRK1. Consistent with our previous studies (2), we observed that wild-type ZBRK1 repressed transcription from a reporter template bearing multimerized ZREs located upstream of the HSV TK promoter in U2OS cells at least 10-fold in a dose-dependent manner (data not shown). To examine the influence of the ZBRK1 CTRD on repression directed by wild-type ZBRK1, we transfected a sub-optimal nanogram quantity of the ZBRK1 expression vector that supports only ~2.5-fold repression of transcription from this reporter template (Fig. 6*B*). Under these conditions, ectopic expression of GAL4-CTRD enhanced this level of repression up to 4-fold in a dose-dependent manner (Fig. 6*B*). Importantly, GAL4-CTRD had minimal influence on reporter activity in the absence of ZBRK1, confirming that the CTRD potentiates ZBRK1 repression in this assay by virtue of its direct recruitment to ZBRK1 and not to DNA (Fig. 6*B*). Furthermore, an oligomerization-defective CTRD mutant (GAL4-CTRD ΔC1) bearing a nine amino acid deletion from the CTRD C terminus was completely defective in its ability to potentiate ZBRK1 repression in this assay, confirming that CTRD-mediated oligomerization is a requirement for its ZBRK1 co-repressor activity (Fig. 6*C*). Collectively, these experiments reveal an unanticipated dual function for ZBRK1 in both DNA binding-dependent and -independent modes of repression, the latter of which derives from the ability of ZBRK1 to homo-oligomerize through its CTRD.

DISCUSSION

The identification of ZBRK1 itself during an unbiased search for ZBRK1 CTRD co-regulators prompted us to characterize the structural requirements for and the functional consequences of ZBRK1 homo-oligomerization. Herein we demonstrate that ZBRK1 undergoes tetrameric oligomerization both *in vitro* and *in vivo*, and we further identify the ZBRK1 CTRD C terminus to be both necessary and sufficient for tetrameric oligomerization. Functionally, we demonstrate that ZBRK1 oligomerization facilitates ZBRK1-directed transcriptional repression and concomitantly identify several unique and biologically significant features of ZBRK1 relevant to its role as a KRAB-ZFP transcriptional repressor. First, by virtue of its ability to homo-oligomerize, we identify an unanticipated role for ZBRK1 as a DNA binding-independent co-repressor in addition to its well characterized role as a sequence-specific DNA-binding transcriptional repressor (2). In this regard, we identify both the KRAB and CTRD repression domains within ZBRK1 to be necessary for oligomerization-dependent repression. Whether or not these domains are sufficient for oligomerization-dependent repression remains to be established. Second, we reveal the ZBRK1 CTRD to be a novel protein interaction surface responsible for directing both homotypic and the heterotypic interactions necessary for BRCA1-dependent ZBRK1 transcriptional repression.

In general, oligomerization enhances regulatory diversity among sequence-specific DNA-binding transcription factors by expanding the number of potential response elements to which oligomers can effectively bind and/or by increasing the number and type of transcriptional regulatory domains that can effectively function at a core promoter. Presently, we do not know whether and how ZBRK1 oligomerization might influence the choice of DNA sequences recognized by ZBRK1. The 15-base pair consensus ZRE was originally identified by sampling a random pool of double-stranded oligonucleotides with a recombinant ZBRK1 derivative comprised solely of the eight ZBRK1 zinc fingers but lacking the C-terminal extension that we show here to be critical for oligomerization (2). Future studies will be required to determine whether and how oligomerization-competent forms of ZBRK1 preferentially recognize an expanded ZRE. Interestingly, our most recent studies revealed that the inherent DNA binding activity of ZBRK1 zinc fingers 1–7 on a consensus ZRE is constrained by the ZBRK1 C terminus, suggesting a potential role for the ZBRK1 oligomerization domain in regulating the

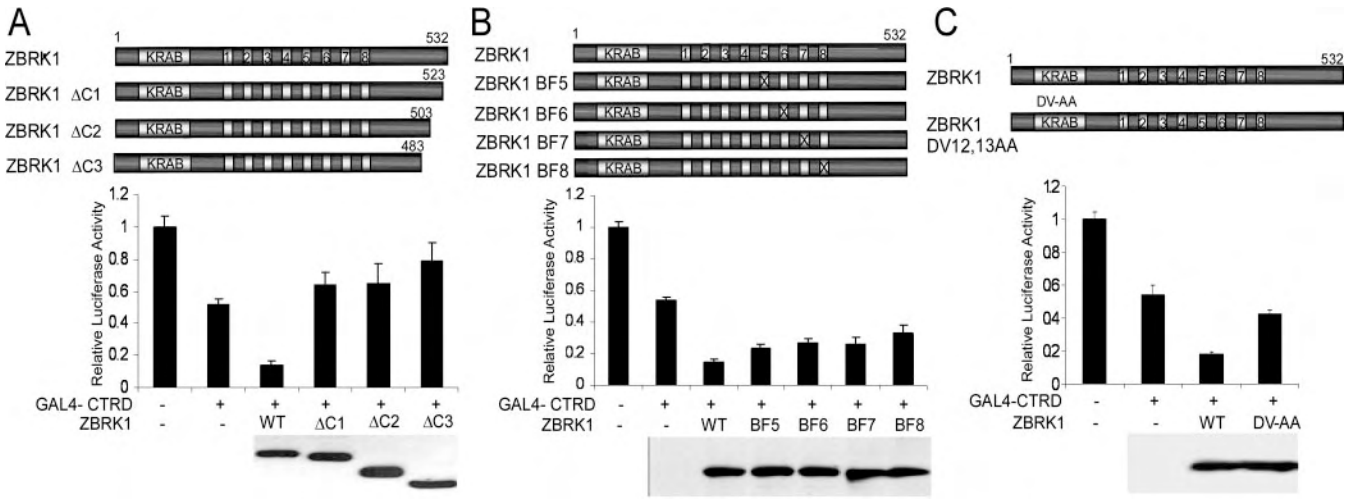

FIG. 5. **Structural requirements for ZBRK1 co-repressor activity.** *A–C*, *top*, schematic representation of FLAG-tagged wild-type ZBRK1 and its ΔC (*A*), BF (*B*), and KRAB domain (*C*) mutant derivatives. *Middle*, U20S cells were transfected with 100 ng of the pG_5TK-luciferase reporter template without or with 600 ng of pM-ZBRK1-CTRD expressing GAL4-ZBRK1-CTRD as indicated and the normalized amounts of pCS2+-FLAG-ZBRK1 expressing FLAG-tagged ZBRK1 or its indicated mutant derivatives (200 ng each of wild-type ZBRK1 (*WT*), ZBRK1 BF derivatives, and ZBRK1 D12A,V13A (*DV-AA*); 100 ng of ZBRK1 ΔC1; 150 ng of ΔC2; and 75 ng of ΔC3). Relative luciferase activities were calculated as described in the legend to Fig. 4. *Bottom*, to confirm that wild-type FLAG-tagged ZBRK1 and its mutant derivatives were expressed at roughly equivalent levels in functional assays, equivalent proportional aliquots of transfected U20S cell lysates used in functional assays (*middle*) were also resolved by SDS-10% PAGE and subjected to immunoblot analysis using FLAG epitope-specific antibodies.

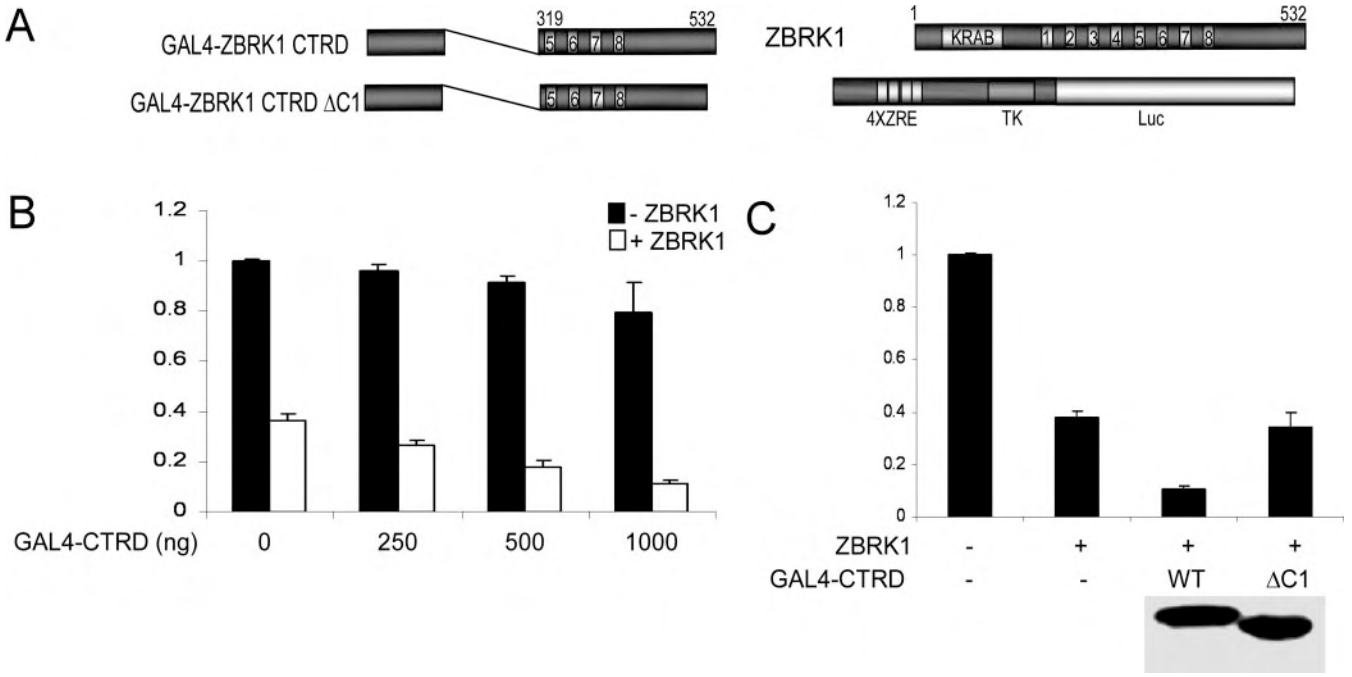

FIG. 6. **CTRD-mediated oligomerization is essential for ZBRK1 co-repressor activity.** *A*, schematic representation of GAL4-ZBRK1-CTRD, GAL4-ZBRK1-CTRD ΔC1, FLAG-tagged ZBRK1, and a $pZRE_4TK$-luciferase reporter (*4XZRE TK Luc*) template used in transfection assays. *B*, U20S cells were transfected with 100 ng of the $pZRE_4TK$-Luc reporter template without or with 800 ng of pCS2+-FLAG-ZBRK1 expressing FLAG-tagged ZBRK1 and the indicated nanogram amounts of pM-ZBRK1 CTRD expressing GAL4-ZBRK1 CTRD. *C*, U2OS cells were transfected with 100 ng of the $pZRE_4TK$-Luc reporter template without or with 800 ng of pCS2+-FLAG-ZBRK1 expressing FLAG-tagged ZBRK1 and normalized amounts of pM-based plasmids expressing GAL4-ZBRK1 CTRD (1000 ng) or GAL4-ZBRK1-CTRD ΔC1 (500 ng). To confirm that wild-type (*WT*) GAL4-ZBRK1 CTRD and GAL4-ZBRK1-CTRD ΔC1 were expressed at equivalent levels in functional assays, equivalent proportional aliquots of transfected U20S cell lysates used in the functional assays were also resolved by SDS-PAGE (10% gel) and subjected to immunoblot analysis using FLAG epitope-specific antibodies.

sequence-specific association of ZBRK1 with a consensus ZRE (13). Although our previous studies clearly revealed that ZBRK1 can bind to and repress transcription from a single ZRE (2, 13), it nonetheless remains to be established whether and how ZBRK1 oligomerization might facilitate synergistic binding and/or repression from multimerized ZREs.

Our work reveals a unique and unanticipated dual function for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression. With respect to the latter, we demonstrate that ZBRK1 can function as a co-repressor in a manner dependent upon the integrity of its CTRD and KRAB repression domains but not upon its inherent DNA binding activity. This observation raises the possibility that the regulatory potential of ZBRK1 on certain target genes may derive from its recruitment via protein-protein rather than protein-DNA interactions. To our knowledge, this represents the first

example of a KRAB-ZFP that can function dually as both a DNA-binding repressor as well as a DNA binding-independent co-repressor. Thus, we propose that higher order oligomers of ZBRK1 may assemble on a target ZRE through protein-DNA and protein-protein interactions, the latter involving oligomerization through the CTRD C terminus.

Our work further provides unique insight into the structural and functional organization of ZBRK1 as a member of the KRAB-ZFP family. Like other KRAB-ZFPs, ZBRK1 harbors an N-terminal KRAB repression domain followed by a C_2H_2 zinc finger DNA-binding domain. However, ZBRK1 also harbors an atypical C-terminal extension that is generally absent from the larger family of KRAB-ZFPs. Our previous work revealed this C-terminal extension to comprise part of a novel BRCA1-dependent CTRD that also includes zinc fingers 5–8 within ZBRK1 (13). Herein, we describe an additional and unique role for the CTRD as an oligomerization interface sufficient for directing homotypic interactions critical for ZBRK1 repressor function. Within the CTRD we demonstrate that zinc fingers 5–8 are dispensable, whereas the C-terminal extension is essential for ZBRK1 oligomerization. This C-terminal extension shows no obvious sequence similarity to the SCAN domain that mediates selective oligomerization between certain KRAB-ZFPs (1, 18). However, a BLAST search of available protein databases reveals several additional KRAB-ZFPs (ZNF577, ZNF613, and FLJ12644), each of which carries an extended C terminus of variable length with sequence homology to the ZBRK1 C-terminal extension. Interestingly, these proteins exhibit the same rank order with respect to their degree of homology and physical proximity to ZBRK1 on chromosome 19q, suggesting that they may have arisen from gene duplication (19–21). It will be of future interest to determine whether and how these KRAB-ZFPs functionally oligomerize with ZBRK1 and/or BRCA1. Although zinc fingers 5–8 within the CTRD are dispensable for oligomerization, our previous studies have nonetheless shown them to be essential for mediating interactions with additional co-repressors, including BRCA1 (2, 13). Thus, our studies collectively reveal the CTRD to be a novel protein-interaction surface responsible for directing both homotypic and heterotypic interactions necessary for BRCA1-dependent ZBRK1 transcriptional repression.

Finally, our identification of a novel oligomerization domain within ZBRK1 suggests that the regulatory potential of ZBRK1 could extend to genes that do not contain ZREs. For example, ZBRK1 could repress transcription through its hetero-oligomerization with other sequence-specific DNA binding transcription factors through direct protein-protein interactions involving its C terminus. This, in turn, could expand the repertoire of target genes subject to coordinate transcriptional control by both ZBRK1 and BRCA1, because the repressive potential of ZBRK1 is dependent upon BRCA1 (2, 13). Consistent with this possibility, our yeast two-hybrid screen for CTRD-interacting proteins identified, in addition to ZBRK1 itself, the oligomeric transcription factors SRF and ATF-1 (22–26). Interestingly, these transcription factors control the expression of genes with diverse functions in cell growth control and survival, and future studies will seek to establish whether and how ZBRK1 physically and functionally interacts with these transcriptional regulators. Nonetheless, our work here sheds new light on the structural and functional organization of ZBRK1 as a KRAB-ZFP and further defines the multifunctional nature of its unique CTRD.

Acknowledgements—We thank our laboratory colleagues for advice, discussion, and comments.

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BRCA1 and *BRCA2* in breast cancer

Wen-Hwa Lee, Thomas G Boyer

The inheritance of an autosomal dominant allele represents an identifiable predisposing factor in about 10% of all women with breast cancer. Most of these hereditary cases can be linked to germline mutations in either of two breast cancer susceptibility genes, *BRCA1* or *BRCA2*. Women who have a mutation in either of these genes have a cumulative lifetime risk of 60–80% and 20–40% for the development of breast and ovarian cancer, respectively. There is therefore a great need for new and effective measures for their management. Progress in our understanding of the normal biological function and regulation of *BRCA1* and *BRCA2* has shed new light on the molecular basis of hereditary breast cancer, and should provide a driving force for the development of diagnostic and therapeutic strategies.

BRCA1 and *BRCA2* are caretaker genes whose products function in the maintenance of global genome stability—ie, they ensure that the genetic integrity of a cell is not compromised by the unscheduled loss, duplication, or rearrangement of chromosomal DNA. A persistent threat to genome integrity is DNA damage arising from ongoing metabolic processes within the cell, as well as that elicited by extrinsic agents, including radiation and certain chemicals. Unrepaired or misrepaired DNA damage can compromise chromosomal stability, allowing a cell to escape normal restrictions on its growth.

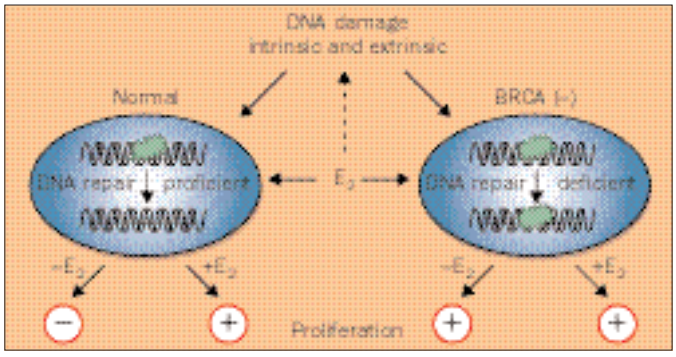
Genome integrity is ensured in part by a response system that has evolved to locate and effect the timely repair of damage to DNA. This response involves the assembly of DNA-repair protein complexes able to recognise and eliminate damage-induced lesions, and the synthesis of cell-cycle checkpoint control proteins that provide a sufficient window of opportunity to effect such repair. *BRCA1* and *BRCA2* occupy fundamental roles in coupling DNA damage-induced signals to downstream cellular responses, including damage repair and cell-cycle checkpoint activation.

Because the DNA damage-induced signalling pathways that converge on *BRCA1* and *BRCA2* are universally conserved, the genes are likely to function ubiquitously in the maintenance of genome integrity. Nonetheless, inactivation of *BRCA1* or *BRCA2* generally leads only to cancer of the breast or ovary. What then might constitute the molecular basis for the tissue-specific tumour suppressive properties of *BRCA1* and *BRCA2*?

The breast and the ovary are reproductive organs that rely on hormones, including oestrogen and progesterone, for growth, differentiation, and homeostasis. According to one theory, inactivation of *BRCA1* and *BRCA2* renders breast and ovary susceptible to tissue-specific effects of oestrogen-induced DNA damage. Thus, inactivating mutations in *BRCA1* and *BRCA2* could compromise the response of breast and ovarian epithelial cells to oestrogen-induced DNA damage, thereby resulting in inefficient or error-prone DNA repair. Global genomic instability and a concomitant accrual of functionally inactivating mutations within other genes involved in breast and ovarian tumourigenesis might then ensue. Alternatively, *BRCA1* might modulate hormone signalling pathways and control of cellular proliferation. *BRCA1* represses the transcriptional activity of the oestrogen and progesterone receptors, and mutational inactivation of the gene could, therefore, promote epithelial cell proliferation through altered expression of hormone-responsive genes.

These two models are not mutually exclusive and could suggest a combinatorial path to breast cancer, since they invoke *BRCA1*-mediated and *BRCA2*-mediated control at two distinct steps of tumourigenesis—initiation and progression. Thus, inappropriate expression of hormone-responsive genes could promote the proliferation of transformed cells arising through inefficient or error-prone repair of oestrogen-induced DNA damage. In this way, hereditary *BRCA1* and *BRCA2* mutations could render breast and ovarian epithelial cells particularly susceptible to tumourigenesis through perturbation of distinct hormone-dependent pathways (figure).

This knowledge could help to treat those carrying mutations in *BRCA1* and *BRCA2*, and might also be useful in the treatment of patients with sporadic, non-genetic breast cancers. Few mutations in *BRCA1* and *BRCA2* arise in sporadic breast cancers, suggesting that the perturbation of alternative pathways causes malignant disease in these cases. As caretakers of genomic integrity, *BRCA1* and *BRCA2* represent prime targets for therapeutic intervention—ie, targeted inactivation of *BRCA1*-specific and *BRCA2*-specific DNA-damage response pathways could render tumour cells sensitive to the genotoxic effects of radiation or chemotherapeutic agents, thereby offering the potential for improved combination therapies. In the last decade of the 20th century, *BRCA1* and *BRCA2* were identified and characterised. The role and regulation of their encoded products in DNA-damage response and repair, once identified, should expedite the design and implementation of strategies to delay, and ultimately to prevent, tumour formation.



Role of *BRCA* genes in breast cancer
E_2=oestrogen.

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Appendix 8

Breast Cancer Susceptibility Genes

by Thomas G. Boyer and Wen-Hwa Lee

The last decade of the 20th century witnessed the identification and initial characterization of two major breast cancer susceptibility genes, *BRCA-1* and *BRCA-2*. Studies of the encoded BRCA proteins have revealed roles in the maintenance of chromosomal stability and in DNA damage response and repair, and studies continue to illuminate further biological activities. A greater appreciation of the involvement of *BRCA-1* and *2* in breast and ovary cells will increase the probability that recent advances in our understanding of their biological functions will be channeled effectively to the treatment and prevention of breast and ovarian cancer.

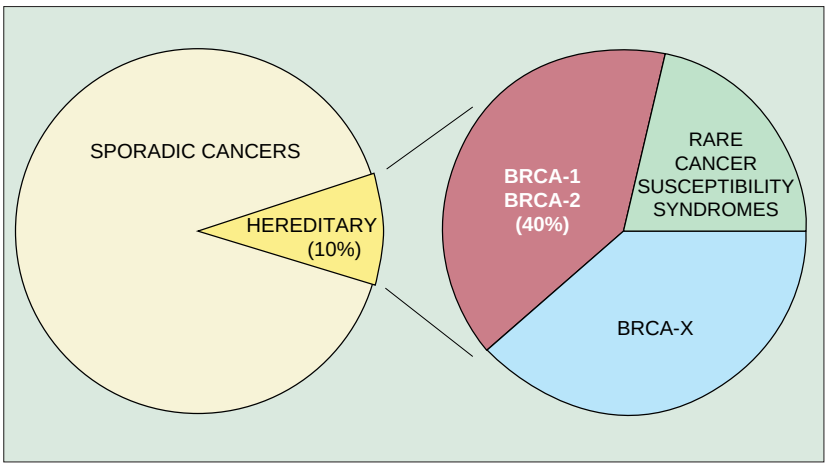
In the year 2002, approximately 200,000 American women will be diagnosed with breast cancer, the most common malignancy afflicting women in the United States. Among women who do not smoke, breast cancer is the primary cause of cancer-related death.

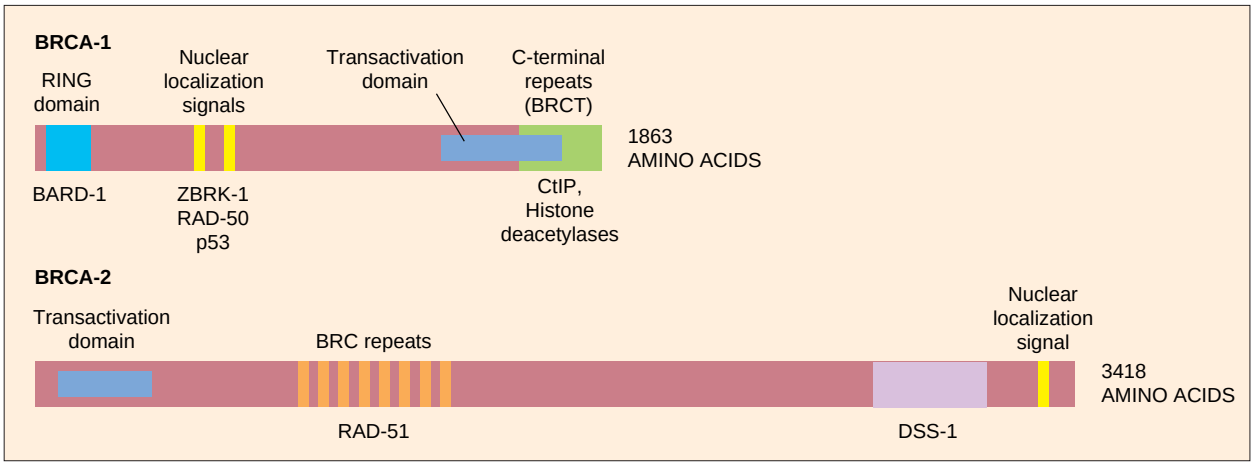
Although many factors influence a woman's lifetime risk for development of breast cancer, family history is one of the most powerful prognostic indicators. About 10% of all breast cancer cases can be linked to heritable transmission of an autosomal dominant allele. Thus a major achievement was substantiation that many of these hereditary cases could be linked to germline mutations in either of two breast cancer susceptibility genes, identified as *BRCA-1* and *2*.

Through linkage analysis of families affected by early-onset breast and ovarian cancer, *BRCA-1* was mapped to chromosome 17q21 in 1990 and cloned 4 years later. *BRCA-2* was mapped to chromosome 13q and cloned shortly thereafter. Mutations in *BRCA-1* are believed to account for 60 to 80% of hereditary breast and ovarian cancer cases and up to 20% of hereditary breast cancers only. *BRCA-2* mutations are linked to a similar percentage of inherited breast cancers, but in contrast to *BRCA-1*, they also predispose to male breast cancer.

Together, defects in these two genes account for about 40% of inherited breast cancers. Germline inactivation of one allele of either *BRCA-1* or *2* is sufficient to predis-

Mutations in BRCA genes account for almost half of hereditary breast cancers. The rest derive from mutations either in identified genes associated with rare cancer susceptibility syndromes or in unidentified susceptibility genes.





Structural and functional domains of BRCA-1 and 2 are named above each of the schematic proteins. Representative proteins that interact with BRCA-1 and 2 are identified beneath them.

pose a person to cancer, while cancer onset is invariably accompanied by loss of the remaining allele. Thus *BRCA-1* and *2* belong to the group of tumor susceptibility genes whose encoded products normally function to suppress tumor formation.

Mutations in other known tumor susceptibility genes, such as *p53*, the retinoblastoma gene *RB*, and the adenomatous polyposis gene *APC*, are found in both familial and sporadic tumors. Mutations in *BRCA-1* and *2*, however, are rarely detected in nonhereditary breast cancers, though it has been proposed that aberrant regulation of their expression or of the activity of their products could contribute to sporadic breast cancers.

Clearly, detailed knowledge of the normal biological functions of these proteins and of their regulation will be required for a thorough appreciation of how direct or indirect functional inactivation of *BRCA-1* and *2* leads ultimately to breast cancer. In this article, we begin with a description of the structural features of the BRCA proteins and then highlight recent insights into their biological role and regulation.

Protein Structures Are Clues to Functions

BRCA-1 is a nuclear phosphoprotein of 1863 amino acids characterized by the presence of a notable structural motif near each end.

At its amino terminus, BRCA-1 harbors a zinc-binding RING finger domain, which is a set of spatially conserved cysteine and histidine residues. More than 200 RING finger proteins of diverse function are potentially encoded by the human genome, so this domain is a relatively common structural motif. Recent studies have raised the possibility that the functional diversity apparent among RING finger proteins is tied to a common enzymatic activity.

The carboxy terminal of BRCA-1 includes a series of domains that are autonomous folding units defined by conserved clusters of hydrophobic amino acids. These are called BRCA-1 C-terminal or BRCT domains, and they have been found in other proteins implicated in DNA repair and cell cycle checkpoint control. No specific cellular function has so far been ascribed to the BRCT domain, but it is likely to be a protein interaction surface.

A third region in BRCA-1 also appears to be a functionally relevant protein interaction surface, but the structure of this region has not yet been defined. The same region includes two putative nuclear localization signals.

BRCA-2 is a nuclear protein of 3418 amino acids whose most prominent feature is eight tandem copies of a repetitive sequence termed the BRC repeat. Also notable is a region of about 500

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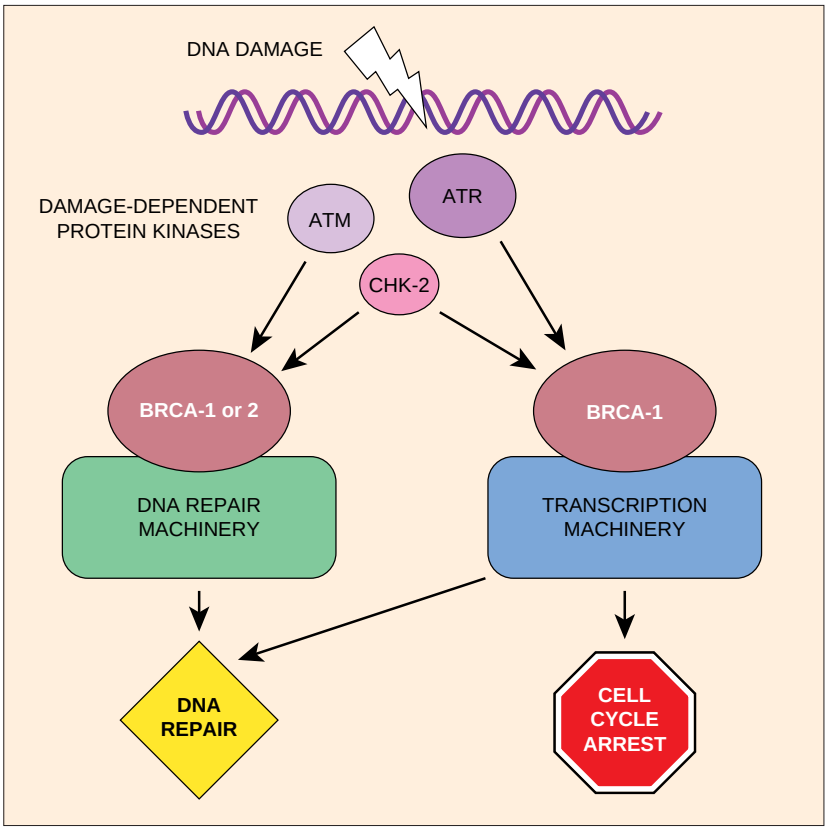
are in the Department of Molecular Medicine and Institute of Biotechnology, University of Texas Health Science Center at San Antonio.



BRCA-1 and 2 couple signals of DNA damage to cellular responses, including damage repair and cell cycle checkpoint activities. Though much about this process is not yet understood, it is known that BRCA-1 is phosphorylated by any of several protein kinases, depending on the type of DNA damage. Both BRCA proteins interact physically with DNA repair proteins, and BRCA-1 also participates in transcription control of genes that encode DNA repair and cell cycle checkpoint control proteins.

amino acids that is more highly conserved between human and mouse than the coding sequence as a whole.

BRCA-1 and 2 Are Caretakers of Genomic Stability

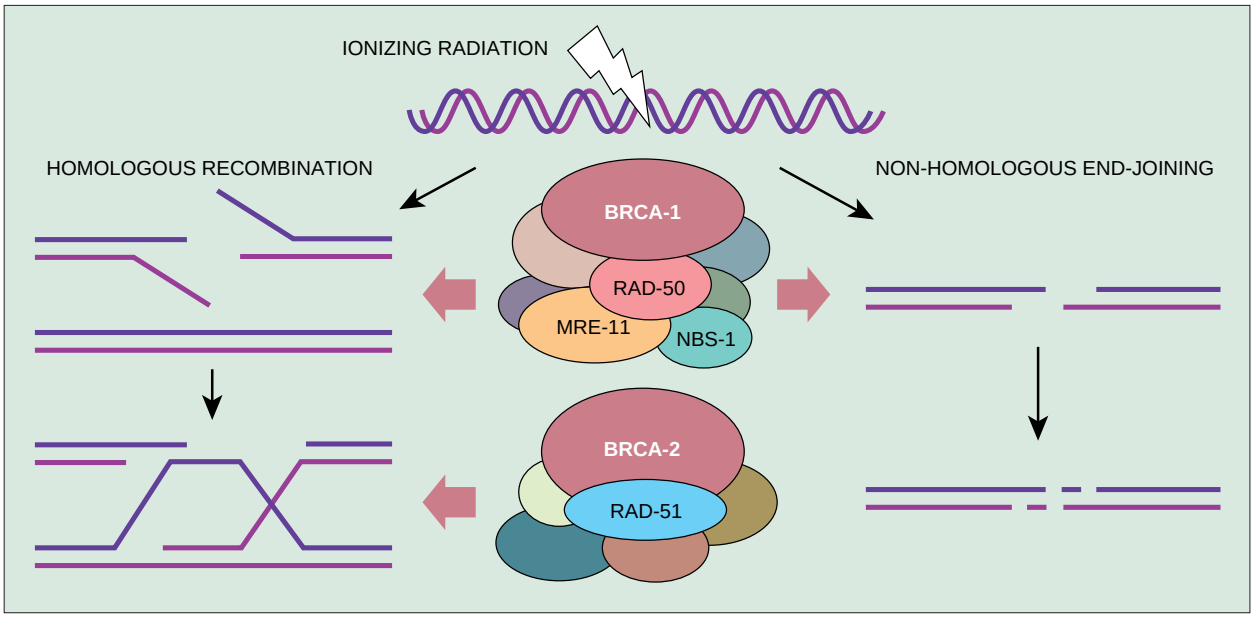
Insights into the biological functions of BRCA-1 and 2 have come from analyses of cells derived from BRCA-mutant human breast tumors and from embryos of mice carrying targeted deletions of the *BRCA* genes. Invariably, BRCA-deficient cells exhibit gross chromosomal abnormalities, typified by breaks, aberrant mitotic exchanges, and aneuploidy.

These sorts of DNA damage arising from ongoing metabolic processes within the cell or caused by extrinsic agents, including radiation and certain chemicals, are a persistent threat to genome integrity. A response system has evolved to locate damaged DNA and effect its timely repair. BRCA-1 and 2 are parts of that system, cellular caretakers ensuring that the genetic integrity of a cell is not compromised by the unscheduled loss, duplication, or rearrangement of chromosomal DNA.

The DNA repair response involves the assembly of protein complexes capable of recognizing and eliminating damage-induced lesions, as well as the synthesis of proteins that arrest cell cycle progression while the damage is repaired. Disruption of the damage response system can lead to replication or segregation of damaged chromosomal DNA, and that in turn can permit a cell to escape normal restrictions on its growth, which is practically the definition of cancer.

Evidence to implicate BRCA-1 and 2 in the DNA damage response has come from the observation that cells deficient in either protein are hypersensitive to a variety of DNA-damaging agents. A more specific function was suggested by the finding that cells

Discovery of the BRCA genes and early work on their protein products was described by Barbara Weber in the January/February 1996 issue of SCIENCE & MEDICINE.



DNA double-strand breaks induced for example by ionizing radiation are repaired by two processes in which the BRCA proteins are involved. In a complex with RAD-50, MRE-11, NBS-1, and other proteins, BRCA-1 takes part in both homologous recombination and nonhomologous end-joining. BRCA-2 complexed with RAD-51 is active in strand exchange during homologous recombination.

deficient in either BRCA-1 or 2 exhibited overt defects in the repair of oxidative DNA damage. Further studies have documented direct interactions between BRCA-1 or BRCA-2 and individual protein components of the DNA repair machinery.

BRCA-1 has been linked to DNA repair through its interaction with a complex of three proteins, RAD-50/MRE-11/NBS-1, that operates in both nonhomologous and homologous recombinational repair of double-strand breaks. The three-protein complex has been proposed to resect DNA ends at the sites of double-strand breaks in order to reveal sequence homologies through which recombination can ensue. What BRCA-1 does in its association with this complex remains to be established.

BRCA-1 is also a resident component of a large multiprotein complex that includes mismatch repair proteins. These and other proteins are involved in replication or in repair of DNA damage that can occur at replication forks. The association of BRCA-1 with these proteins suggests that it participates in resolving aberrant DNA structures that appear during replication or when replication is stalled.

A role for BRCA-2 in DNA damage repair has been suggested by the discovery that it interacts with a recombinase called, in mammals, RAD-51. Mammalian RAD-51 is a homologue of the prokaryotic RecA and yeast Rad51p proteins, the latter a member of the RAD-52 epistasis group. In yeast, RAD-52 epistasis proteins are required for repair of DNA double-strand breaks as well as mitotic and meiotic recombination.

Eukaryotic RAD-51 proteins, like RecA, have intrinsic ATP-dependent DNA binding activity. RAD-51 and single-strand DNA form a nucleoprotein filament that invades and pairs with a homologous DNA duplex, catalyzing homologous DNA pairing and strand exchange. Mouse embryos lacking BRCA-2 exhibit radiation hypersensitivity defects like those seen in mouse embryos lacking RAD-51.

The interaction between BRCA-2 and RAD-51 involves the BRC repeats in BRCA-2. Peptides corresponding to individual BRC repeats can inhibit multimerization of RAD-51 and block nucleoprotein filament formation. Whether this inhibitory activity is a physiological role for BRCA-2 in regulating RAD-51 activity has not been established.

AUTHOR--
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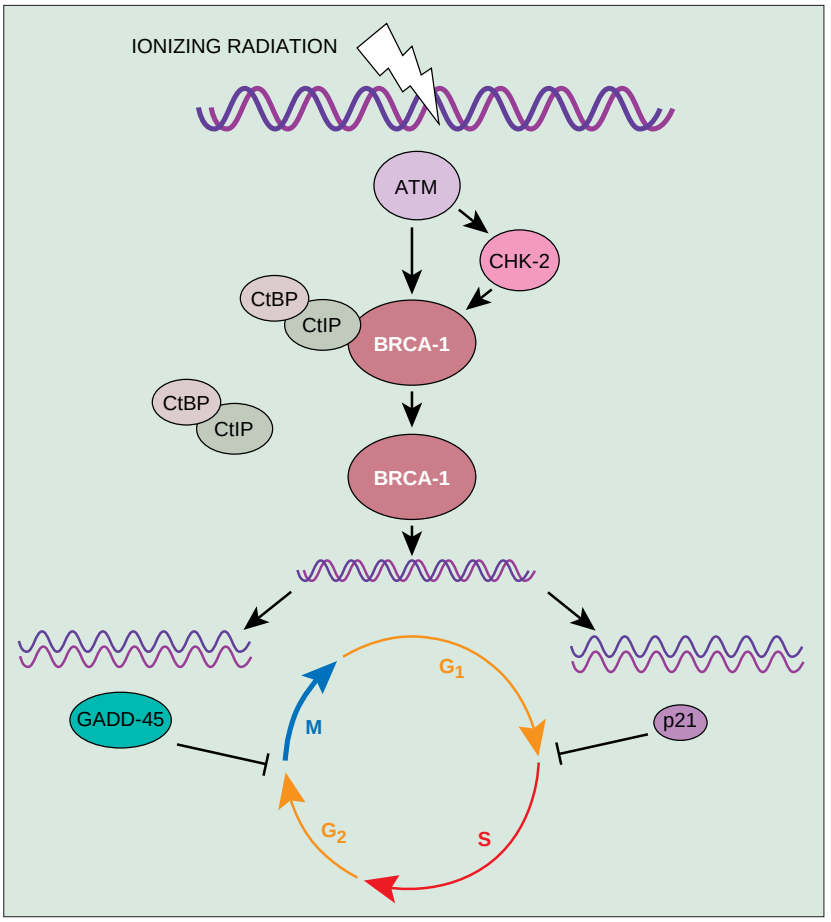


With associated co-repressor CtIP, BRCA-1 ordinarily represses transcription of cell cycle checkpoint control genes. In response to certain kinds of DNA damage, BRCA-1 and CtIP are phosphorylated by ATM. Phosphorylated CtIP dissociates from BRCA-1, leading to relief of BRCA-1-mediated transcriptional repression and consequent induction of *p21* and *GADD-45*. The protein products of those genes function in G_1-S and G_2-M cell cycle arrest, respectively.

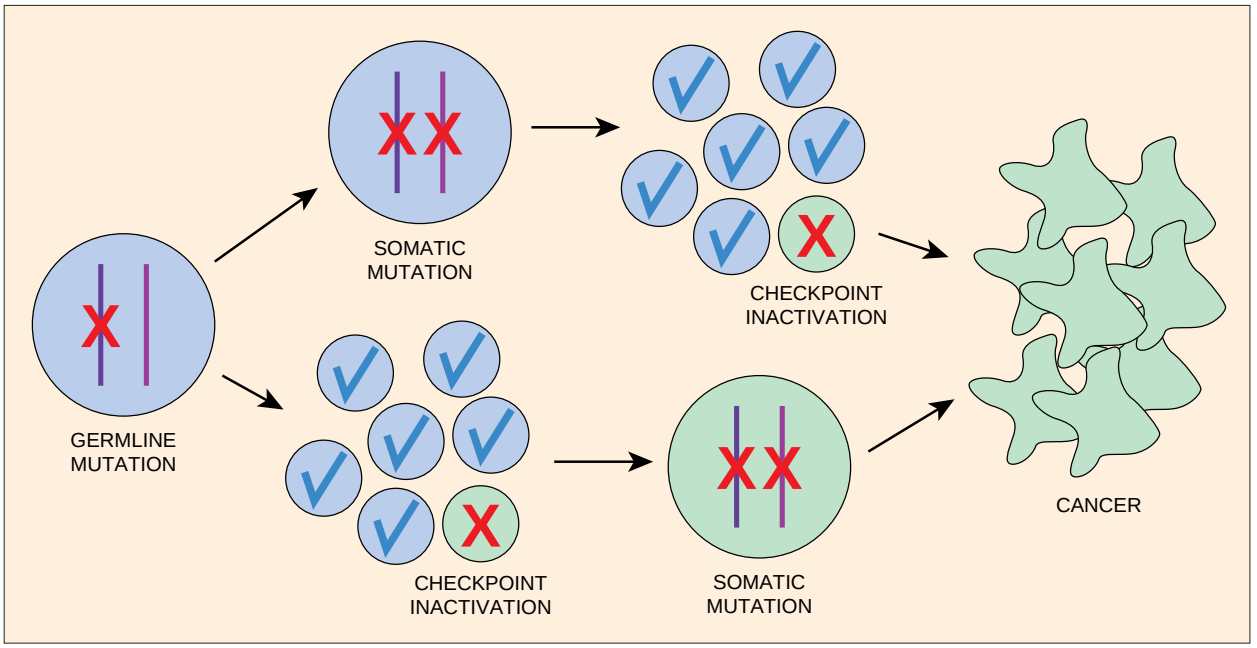
Consistent with that possibility, though, is the observation that formation of RAD-51 protein complexes, normally induced by DNA damage, is diminished in cells either deficient in BRCA-2 or in which the interaction between BRCA-2 and RAD-51 is specifically disrupted. It therefore seems clear that BRCA-2 is necessary for the assembly of RAD-51 complexes.

Processes of DNA repair must be coordinated with regulation of cell cycle transit so that damage is repaired before chromosomal DNA is replicated or segregated. There is considerable evidence that BRCA-1 occupies a central place in activation of cell cycle checkpoints when DNA damage is detected.

First, BRCA-1-mutant cells exhibit defects in DNA damage-induced S and G_2-M cell cycle checkpoints. Second, after DNA damage, BRCA-1 is rapidly phosphorylated by cell cycle checkpoint kinases, suggesting that it functions downstream of DNA damage sensors that trigger cell cycle checkpoints. And third, BRCA-1 has been shown to regulate expression of cell cycle checkpoint control genes, including *p21* and *GADD-45*, which function in G_1-S and G_2-M cell cycle checkpoints, respectively.

The role of BRCA-2 in cell cycle checkpoint control is much less clear. Where examined, DNA damage-induced cell cycle checkpoints appear to be largely intact in cells lacking wild-type BRCA-2. Indirect evidence exists to link BRCA-2 to G_2-M control, but it is not entirely clear whether this is an indirect effect secondary to the role of BRCA-2 in DNA damage repair.

The fact that specific disruption of the interaction between BRCA-2 and RAD-51 leads to loss of G_2-M checkpoint control suggests that this may be the case. Thus, BRCA-



Possible pathways to breast cancer in women carrying germline mutations in *BRCA-1* or *2*.

Somatic inactivation of the remaining allele gives rise to repair-deficient cells. Most of these cells ultimately die because of cumulative DNA damage and activation of cell cycle checkpoints. Rare repair-deficient cells (*green cells*) that survive can acquire additional mutations in cell cycle checkpoint control genes. These cells can survive in spite of genomic instability and give rise to tumors.

Alternatively, mutational inactivation of a cell cycle checkpoint gene may precede somatic inactivation of the remaining *BRCA* allele, resulting in repair deficient cells that can survive despite genomic instability. The result is the same.

Author -- Were confused by the meaning of the colors in the above figure. We will revised the above figure as needed.

2 inactivation could trigger existing checkpoints that monitor DNA structure, leading to delays in G_2-M progresson.

Collectively, the phenotypic characteristics of cells deficient in BRCA-1 or 2 suggest that these proteins are fundamental in the DNA damage response by participating in damage repair, cell cycle checkpoint control, or both.

Chromosomal instability arising from a defective DNA damage repair response has been proposed as the pathogenic basis for tumorigenesis accompanying BRCA deficiency. Paradoxically, chromosomal instability should lead to cell growth arrest or increased cell death, so the question is how BRCA-1 or 2 mutations might lead to the opposite effect.

One answer might lie in the observation that tumor cells deficient in BRCA-1 or 2 frequently harbor other inactivating mutations in cell cycle checkpoint control genes, including *p53*. Those mutations may circumvent the growth arrest that is normally induced by DNA damage and also inhibit p53-mediated apoptosis, permitting the survival of cells despite severe chromosomal damage.

On the other hand, inactivation of mitotic checkpoint genes could bypass mitotic arrest and permit aberrant chromosomes to segregate into progeny cells. This model is supported by experimental observations and suggests that the genetic instability arising in BRCA-1- or 2-deficient cells is pivotal in tumorigenesis, leading first to compensatory gene mutations that override damage-induced cell cycle arrest and apoptosis and subsequently to the accrual of functionally inactivating mutations of genetic loci involved in breast tumorigenesis.

BRCA-1 and 2 Regulate Cell Growth and Differentiation

Emerging evidence suggests important roles for BRCA-1 and 2 in the control of cell growth and differentiation. The clearest example is the observation that homozygous deletion of Brca-1 in mice results in early embryonic lethality accompanied by developmental retardation and cellular proliferation defects.

This outcome can be explained in part by the involvement of BRCA-1 and 2 in DNA repair,

Author--
Please differentiate *BRCA* vs BRCA vs Brca for me. My understanding is that the first is the gene (ital), then protein (no ital), so I don't understand what Brca (both ital and non-ital) represents.

Author--
See figure on opposite page: in your original drawing for activation, you included an arrow pointing to the "bend" in the DNA; in fact, damage should occur at TATA, which in your drawing is covered by the BRCA complex -- is this correct?
Do we need to move the BRCA molecules to cover TATA or re-insert the arrow?

because targeted deletions in *p53* or its downstream effector *p21* can rescue embryos with homozygous Brca-1 and 2 deficiency. Thus, cumulative DNA damage arising in the absence of Brca-1 and 2 has been hypothesized to trigger p53-mediated cell cycle arrest and apoptosis in the developing embryo, while inactivation of *p53* leads to cell cycle checkpoint bypass and survival.

However, inactivation of *p53* only partially rescues these embryos, which survive for only days longer in development. While the delayed embryonic lethality accompanying inactivation of *p53* has been ascribed to the accumulation of gross chromosomal defects that are incompatible with life, the possibility also exists that Brca-1 and 2 are required for transit through a critical point later in embryonic development.

Another line of evidence has come from studies of transgenic mice carrying a *Brca-1* allele that can be targeted for conditional inactivation specifically in the mammary glands of female mice. That inactivation elicits defects in ductal morphogenesis and also induces tumors that are associated with genetic instability, aneuploidy, and chromosomal rearrangements.

In addition to independently supporting a role for BRCA-1 as a breast tumor suppressor, this mouse model has revealed that BRCA-1 is critical in mammary epithelial development. Conditional inactivation of *BRCA-2* specifically in mammary gland has yet to be achieved, so the role of BRCA-2 in mammary gland formation remains to be established.

BRCA-1 and 2 Regulate Transcription

In parallel with the genetic studies, biochemical and molecular biological analyses have been carried out to determine how BRCA-1 and 2 execute their functions. The proteins have been linked to a variety of biological activities.

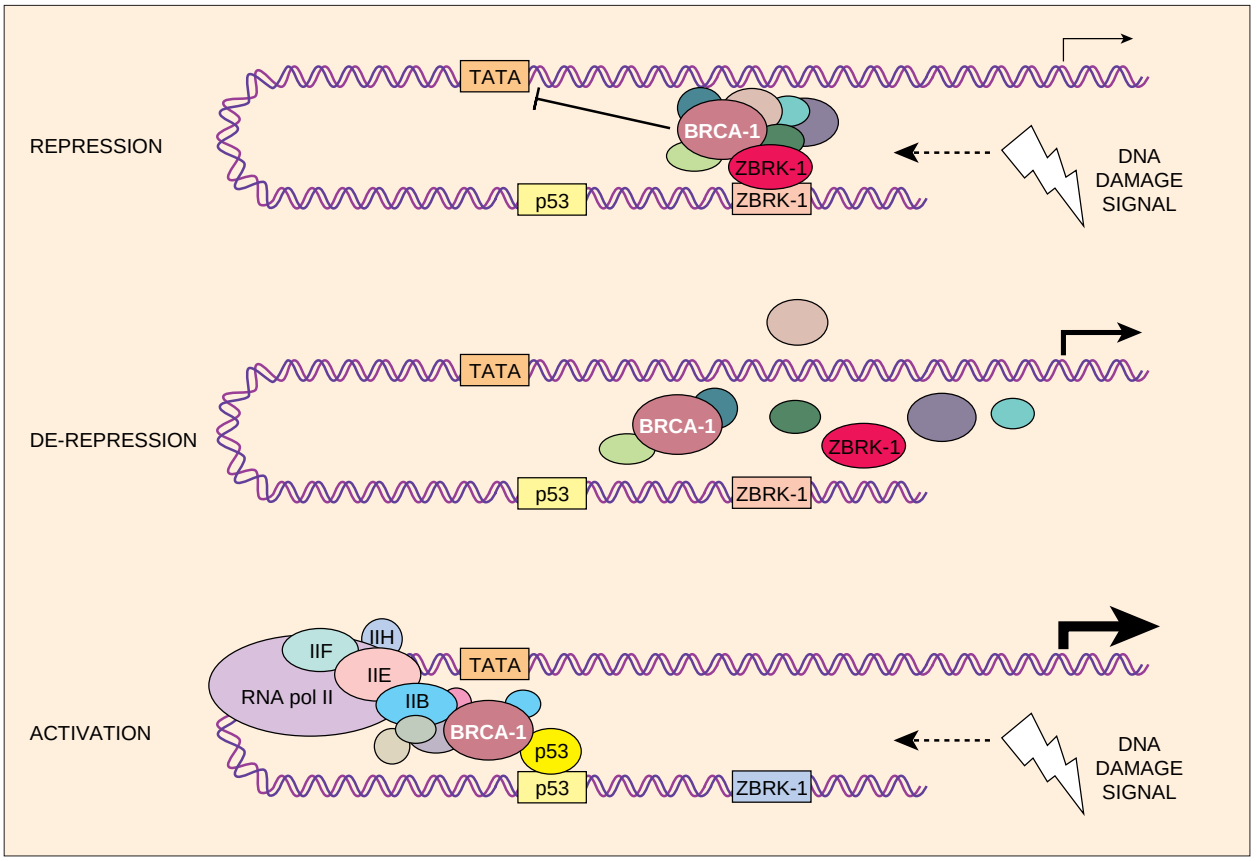
Involvement of BRCA-1 in transcriptional regulation was initially indicated by the identification near its carboxyl terminus of an acidic domain with an inherent transactivation function that is sensitive to cancer-predisposing mutations. This region interacts directly or indirectly with a variety of transcriptional co-activators, including the histone acetyltransferase p300 and hBRG-1, which is the catalytic subunit of a chromatin-remodeling complex called SW-1/SNF.

The same region, interestingly, also interacts with transcriptional co-repressors, including histone deacetylases and the CtIP/CtBP protein complex. *BRCA-1* mutations found in familial breast cancer compomise the trans-activation function but also abolish the binding of BRCA-1 to co-repressors. These observations have prompted the speculation that BRCA-1 may function like a nuclear receptor, either activating or repressing transcription depending on associated co-factors.

Gene expression profiling methods have disclosed that ectopic overexpression of BRCA-1 can induce or repress many genes implicated in cell cycle control, cell cycle regulation, and DNA replication and repair. By virtue of this transcriptional regulatory activity, BRCA-1 could influence cellular responses downstream of DNA damage signals, including DNA repair and cell cycle checkpoint activation.

BRCA-1-mediated regulation of *GADD-45* transcription illustrates how BRCA-1 might participate in cell cycle checkpoint control and also provides a model for how BRCA-1 can achieve gene-specific transcriptional regulation. *GADD-45* is a tumor suppressor gene induced by DNA damage. Its encoded product functions in G_2-M cell cycle checkpoint control.

Induction of *GADD-45* transcription in response to ultraviolet radiation and radiomimetic agents has been shown to depend on BRCA-1, and evidence exists to



suggest that the same may be true for ionizing radiation. It is also known that BRCA-1 interacts with a co-repressor, CtIP, to repress transcription of *GADD-45* and that this interaction is disrupted by DNA damage.

Neither BRCA-1 nor CtIP can bind DNA in a sequence-specific manner, however, so how these proteins are recruited to their target genes was an unresolved question. The answer was recently provided by identification of an intervening protein, named ZBRK-1, that binds to both BRCA-1 and a specific DNA sequence element present in a subset of BRCA-1's target genes, including *GADD-45*.

In this way, BRCA-1 can be physically tethered and functionally linked to specific regulatory loci. It is ZBRK-1 that actually represses transcription when it is bound to BRCA-1, so that BRCA-1 itself is a co-repressor. Potential ZBRK-1 binding sites have been identified in a large group of genes inducible by DNA damage, so the ZBRK-1/BRCA-1 complex may be a global regulator of DNA damage-responsive genes.

A model has been proposed whereby ZBRK-1, BRCA-1, and CtIP coordinately repress a functionally diverse group of DNA damage-response genes in the absence of genotoxic insult, and that phosphorylation induced by DNA damage disrupts the network of interactions among these proteins, de-repressing transcription.

It must be emphasized that de-repression as an operative mechanism in transcriptional control of *GADD-45* and other inducible genes in vivo is likely to be coordinated with other mechanisms of gene activation. BRCA-1 has been reported to interact functionally with a variety of sequence-specific DNA-binding transcriptional activators, including the tumor suppressor p53.

Model for sequence-specific transcription control by BRCA-1 through its dual role as a co-repressor and a co-activator.

ZBRK-1 is a transcriptional repressor that recruits BRCA-1 to its specific DNA binding sites in target genes, one of which is in intron 3 of *GADD-45*. BRCA-1 may then (1) recruit CtIP and CtBP to reorganize higher chromatin structure, (2) recruit histone deacetylase complexes to effect local gene silencing, or (3) interact with the basal transcription machinery.

In response to an appropriate DNA damage signal, BRCA-1-mediated repression of *GADD-45* transcription is relieved. That permits BRCA-1 to become a co-activator of, for example, p53, which also binds to intron 3 of *GADD-45*. BRCA-1 could mediate transcriptional activation by either (1) recruiting chromatin-modifying activities to faciliate transcription complex assembly at the promoter or (2) directly recruiting the RNA polymerase II holoenzyme to the promoter. In this model, damage-induced transcription of *GADD-45* results from concerted de-repression and activation.

In this regard, p53 appears to be an important link between BRCA-1 and transcriptional activation of DNA damage-inducible genes. It lies at the heart of a cell-signaling pathway that is triggered by genotoxic stresses, including DNA damage. Stress-induced p53-initiated cell cycle arrest or apoptosis ensures the timely repair or elimination of potentially deleterious genetic lesions.

Significantly, p53 and BRCA-1 appear to regulate transcription of an overlapping set of DNA damage-inducible target genes, including *GADD-45*. This observation initially implied a functional interaction between these two important tumor suppressors, a prediction that has since been borne out experimentally.

BRCA-1 and p53 have been demonstrated to interact physically and to synergize functionally to activate transcription through a p53-binding site in a *GADD-45* intron. The ability of BRCA-1 to potentiate p53-dependent transcription without itself binding to DNA has led to the hypothesis that BRCA-1 functions as a p53-specific co-activator, possibly linking the biochemical activities of these two proteins to a common pathway of tumor suppression.

By being both a co-repressor and a co-activator of gene transcription, BRCA-1 appears to function as a link between parallel and perhaps synergistic pathways that lead to induction of DNA damage repair effectors. Before it can be understood how BRCA-1 integrates these dual functions, it will be necessary to decipher the mechanistic basis for its independent activation and repression.

In contrast to BRCA-1, the part that BRCA-2 plays in transcriptional regulation is far less certain. Some evidence implicates BRCA-2 in transcription control, including, again, an inherent trans-activation function within the gene that is sensitive to cancer-predisposing mutations and an association with established transcriptional co-factors and histone acetyltransferases. However, the biological significance of these findings has not been demonstrated.

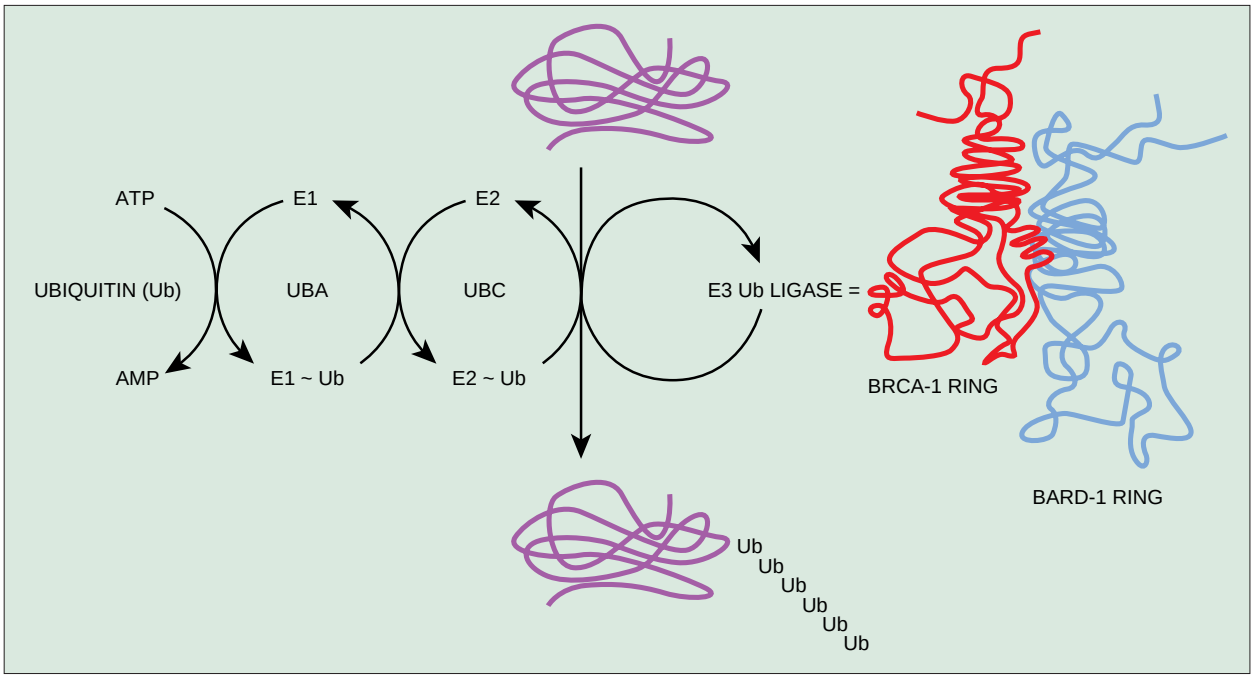
Author--
Can you provide a background paragraph or two on ubiquitination and protein destruction (see underlined paragraph). Our readers aren't (all) cell biologists and would benefit from addtional explanation. This can be inserted into the running text before or after underlined text or shown as a sidebar.

Most if not all of the cellular pool of BRCA-1 resides in stable complexes with other proteins, so one possibility is that BRCA-1 is a molecular scaffold that facilitates assembly of multiprotein machines. Alternatively, the documented association of BRCA-1 with activities that modify chromatin could point to pleiotropic roles in DNA repair and gene transcription. BRCA-1 could variously promote or disrupt nucleosome-mediated condensation of DNA at gene promoters or DNA damage sites, thus precluding or facilitating access by transcription and repair factors, respectively.

Recent work has uncovered a ubiquitin ligase activity of BRCA-1, which raises the intriguing possibility that the protein's multiple functions could all derive from an ability to selectively mark proteins for destruction. Specifically, BRCA-1 interacts with another RING finger protein named BARD-1 through the respective RING domains of each protein. A heterodimeric complex formed by the isolated RING domains of the two proteins exhibits ubiquitin ligase activity in vitro.

Significantly, cancer-related missense mutations within the BRCA-1 RING finger abrogate this activity, suggesting that ubiquitin ligase activity may be important for the biological function of BRCA-1 in breast and ovarian tumor suppression. Presently, no physiological substrates of BRCA-1/BARD-1-targeted ubiquitination have been identified. But if BRCA-1 is involved in targeting proteins for ubiquitination, its participation in a wide range of cellular processes could be explained to some extent.

BARD-1 also interacts with a polyadenylation factor, CstF-50, which indirectly links BRCA-1 to RNA processing. Whether and how the ubiquitin ligase activity of



Ubiquitin is a marker that tags other proteins for destruction. The sequence of events is shown here schematically.

A ubiquitin-activating enzyme E1 (UBA) is charged with ubiquitin, which is then transferred to a ubiquitin-conjugating enzyme E2 (UBC). A ubiquitin ligase E3 presumably functions as a platform for recruitment of both the E2 enzyme and a substrate protein, which is polyubiquitinated and thereby targeted for destruction.

A heterodimer formed by isolated RING domains of BRCA-1 and BARD-1 can function as an E3 ubiquitin ligase in vitro. Remaining surfaces on the two proteins could be involved in substrate recruitment in vivo. The structure of the heterodimer formed by the RING domains has been described.

BRCA-1 alone or in association with BARD-1 contributes to the functions of BRCA-1 is an important area for future investigation.

Tumor Susceptibility Is Tissue-Specific

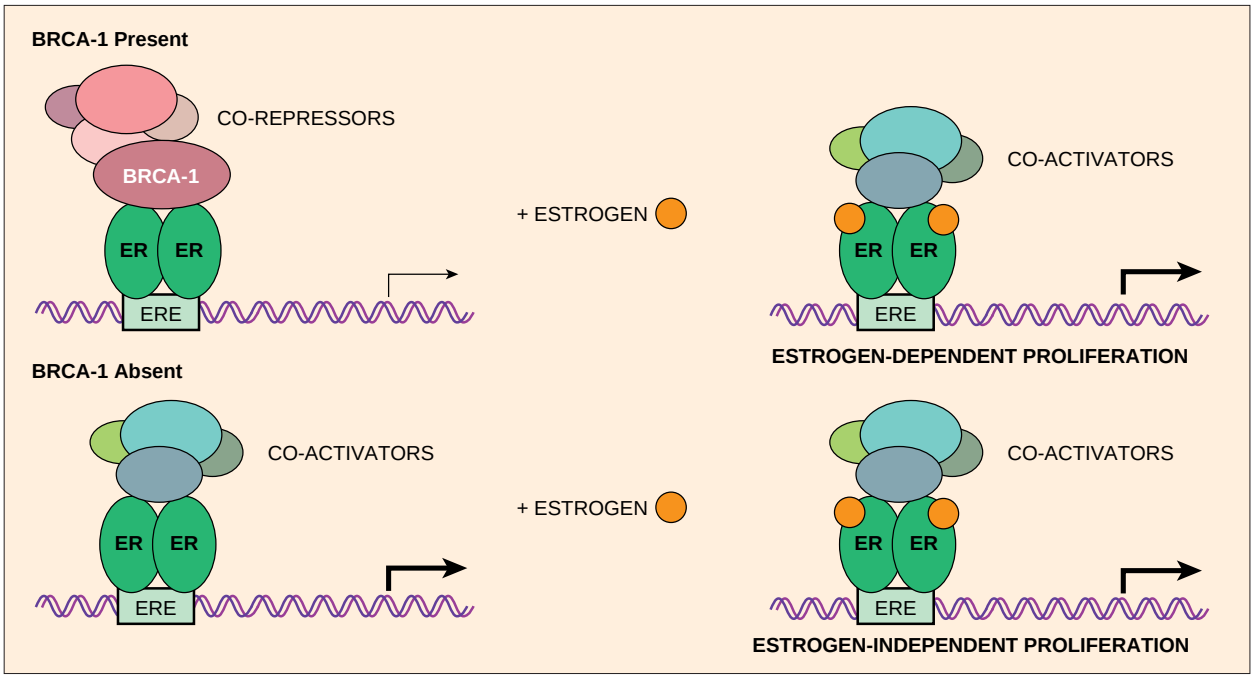
DNA damage response pathways that converge on BRCA-1 and 2 are conserved across many cell types, so that BRCA-1 and 2 are likely to function widely in the maintenance of genomic integrity. Nonetheless, mutational inactivation of these genes leads principally to cancer of the breast and ovary. Why?

As reproductive organs, breast and ovary rely on hormones for growth and differentiation. At least two hypotheses invoking the action of hormones have been proposed to explain the tissue-restricted tumor suppressor functions of BRCA-1 and 2. According to one model, mutational inactivation of the *BRCA* genes renders breast susceptible to the tissue-specific effects of estrogen-induced DNA damage. A major oxidative metabolite of estrogen, 4-hydroxyestradiol, is genotoxic.

The suggestion is that inactivating mutations in *BRCA-1* or *2* could compromise the response of breast epithelial cells in particular to estrogen-induced DNA damage. Inefficient or error-prone DNA repair could then lead to genomic instability and a concomitant accrual of functionally inactivating mutations within other genes involved in breast tumorigenesis. Put another way, *BRCA-1* and *2* mutations might enhance the probability of tumor formation arising from estrogen-induced DNA damage.

A second model proposes that BRCA-1 and 2 modulate hormone signaling pathways that induce cell proliferation. BRCA-1 has been shown to repress the transcriptional activity of the estrogen receptor (ER-α), so mutational inactivation of BRCA-1 could promote epithelial cell proliferation by altering expression of hormone-responsive genes.

The two models are not mutually exclusive and could suggest a combinatorial path to breast cancer, with BRCA-1-mediated control operating at two distinct steps in tumorigenesis, initiation and progression.



BRCA-1 is a barrier to transcription of genes that are targets of the estrogen receptor (ER), preventing cell proliferation by repressing unliganded ER bound to the estrogen response element (ERE). BRCA-1-mediated ER suppression additionally involves one or more co-repressors, minimally including a histone deacetylase activity.

In cells deficient in BRCA-1, ERE-bound ER is free to promote transcription of its target genes and cell proliferation independent of estrogen. Such transcription derives from recruitment of co-activators.

George Kuiper and colleagues discussed the two types of estrogen receptors in the July/August 1998 issue of SCIENCE & MEDICINE.

Models explaining how BRCA-1 acts through modulation of estrogen receptor function must account for the clinical observation that a significant proportion of BRCA-1-associated breast cancers are negative for ER-α expression. A definitive understanding of this phenomenon is precluded by the fact that it simply is not known how "ER-negative" tumors arise.

It has recently been shown that within the terminal ductal lobular unit, where breast cancers are believed to originate, there are at least three distinct epithelial cell populations: ER-α-positive cells that do not proliferative, ER-α-negative cells that do proliferate, and a small number of ER-α-positive cells that can proliferate as well.

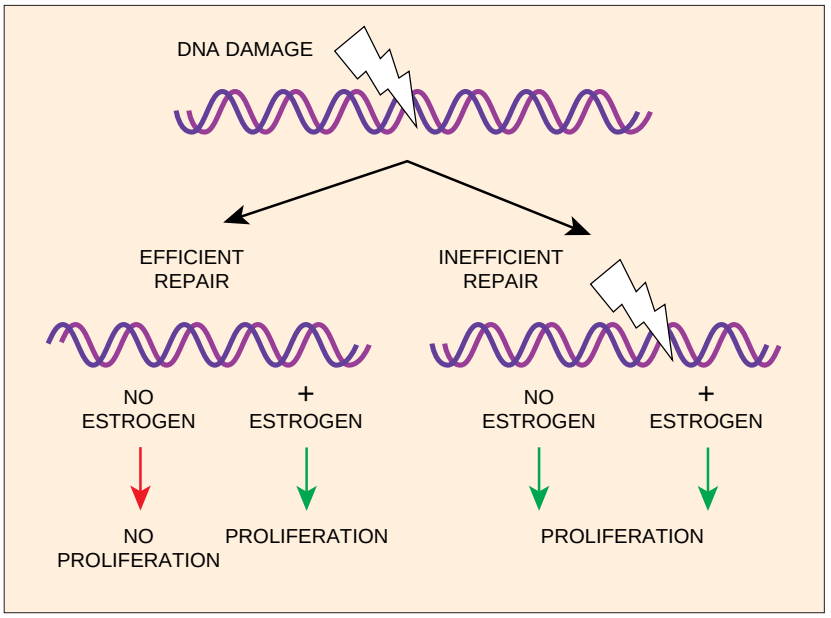
Again, there are two principal models for the genesis of ER-α-negative epithelial-derived tumors, both of which are compatible with a role for BRCA-1 in the control of epithelial cell proliferation through functional interaction with ER-α.

In one model, ER-α-negative breast cancers arise from the loss of ER-α expression during the clinical evolution of cancers that were originally ER-α-positive. In this case, it is possible that the loss of ER-α expression is a relatively late event in breast tumor progression, one that may occur after any proliferative advantages conferred upon transformation-initiated cells by homozygous BRCA-1 mutation have ensued.

Alternatively, it has been proposed that ER-α-negative and ER-α-positive tumors are distinct entities that reflect the receptor status of their clonal origins. Recent data suggest a model in which proliferation of ER-α-negative cells is controlled by paracrine growth factors released from ER-α-positive cells in an estrogen-dependent manner. Here, mutational inactivation of BRCA-1 could promote growth factor-mediated proliferation of ER-α-negative tumors.

Finally, discovery of a second estrogen receptor subtype, ER-β, raises the possibility that this receptor mediates the proliferative response to estrogen in cells that are negative for ER-α expression. ER-β is expressed during the immortalization and transformation of ER-α-negative human breast epithelial cells in vitro.

The functional role of ER-β-mediated estrogen signaling path-



Role of the BRCA proteins in breast cancer. In normal breast epithelial cells, BRCA-1 and 2 ensure efficient DNA repair, thereby preserving genomic integrity in the face of genotoxic insult, including the action of estrogen metabolites. In addition, BRCA-1 restricts estrogen-independent expression of estrogen-responsive genes by directly inhibiting the unliganded estrogen receptor, thus rendering cells dependent on estrogen for growth. BRCA-deficient breast epithelial cells can develop unstable genomes through inefficient repair of damaged DNA and can become independent of estrogen for growth.

ways in the pathogenesis of breast cancer is currently unknown. However, the possibility exists that ER-β may also be subject to BRCA-1-mediated repression.

How might the knowledge now at hand concerning the biological functions of BRCA-1 and 2 be exploited to clinical advantage? For women genetically predisposed to *BRCA-1* and *2* mutations, restricted exposure to direct or indirect extrinsic sources of DNA damage might be warranted.

In reality, knowledge about BRCA-1 and 2 function might find its most useful applications in the treatment of the 90% of sporadic breast cancers for which no genetic linkage with an identifiable susceptibility locus can be found. In these sporadic cancers, pertubation of other pathways are likely involved in tumorigenesis. Nonetheless, as caretaker genes, BRCA-1 and 2 represent prime targets for therapeutic intervention.

For example, targeted inactivation of BRCA-1 and 2-specific DNA damage response pathways could render tumor cells particularly sensitive to the genotoxic effects of radiation or chemotherapeutic agents, offering the potential for improved combination therapies.

Author--
Please list 2 or 3 good recent reviews and a similar number of key primary papers. [the ones below do not need to be kept]

RECENT REVIEWS

Ashok R. Venkitaraman: Cancer susceptibility and the functions of BRCA-1 and BRCA-2. *Cell* 108:171-182, January 25, 2002.

Lei Zheng, Shang Li, Thomas G. Boyer, and Wen-Hwa Lee: Lessons learned from BRCA-1 and BRCA-2. *Oncogene* 19:6159-6175, December 11, 2000.

ORIGINAL PAPERS